

Reasons to be cloned

Current debates on human cloning have been stimulated by questionable achievements. All the more reason for proponents of cloning for biomedical research to articulate the full range of potential benefits.

Human reproductive cloning is widely reviled by cloning experts, who fear that the resulting children will be malformed, if they are born at all. This idea is based on the large number of miscarriages and defective offspring reported in cloned animals. So even without venturing into the debate about whether we would be comfortable having cloned humans for neighbours (or as offspring, or being clones ourselves), the health risks to mother and child inherent in this practice demand that it be banned. But many argue that the prohibitions should stop with reproductive cloning.

The recent announcement by the company Advanced Cell Technology (ACT) in *e-biomed: The Journal of Regenerative Medicine* that it has cloned a human embryo consisting of six cells is regarded as premature by many cloning experts. They say ACT's embryo may be no more than an activated egg, and is devoid of embryonic stem cells. ACT wants to develop cell transplantation therapies targeted to individual patients, by stimulating eggs to form embryos that are genetically identical to adult donor cells. It is anyone's guess whether ACT's announcement was intended to lend transparency to its activities or to publicize them. And the publication has led to a resignation from the journal's editorial board (see page 570). These events highlight the urgency of passing legislation to regulate human cloning.

Stem-cell scientists at ACT and elsewhere need only allow a cloned embryo to develop for five days until it becomes a hollow ball of 100 or so cells. This 'blastocyst' contains no organs or nervous tissue, but possesses a few highly prized embryonic stem (ES) cells, which can be extracted and cultured. ES cells are unique in their ability to continually replenish themselves and, under the right conditions, to form every cell and tissue type in the body.

Such research could fundamentally transform the study of human disease. But whereas the years of culturing embryos in fertility clinics may have brought the first stage of therapeutic cloning — that is, generating ES cells from cloned embryos — closer to hand, scientists are a long way from understanding how these cells can be

grown into organs and tissues for patient transplants. And the economic feasibility of the approach requires scrutiny: there may be other solutions to the problem of graft rejection that would obviate the need to make cloned embryos from patients.

The ability to grow and study ES cells from patients may also further our understanding of why some people get diseases whereas others don't — a notion that hasn't been adequately discussed in the human-cloning debate. Researchers are starting to think about how certain combinations of genetic mutations that occur in our somatic cells and in the germ cells of our parents could predispose us to disease, and influence the age at which we develop the disease and how long we survive. For example, one way to study a disease such as Parkinson's at a molecular level would be to set up ES-cell lines from a patient and from controls, grow them into large quantities of dopaminergic neurons in Petri dishes, and find out why the patient's cells die. The next step would be to determine how to keep them alive. ES-cell cultures cloned from patients could also provide a virtually limitless supply of diseased cells for drug screening and gene-therapy trials.

For such research, it would be essential that work involving human cloning is subject to strict supervision and regulation to ensure that it falls within ethically acceptable boundaries. It may be necessary to address the concern that embryos used in research might lead to the birth of a child, perhaps by developing methods that prevent cloned embryos from ever completing gestation, as ACT is attempting to do by using parthenogenesis to generate embryos.

The US Senate is likely to vote on a bill to ban human cloning when it reconvenes in early January. It is crucial that, before this bill reaches the Senate floor, there is informed debate between scientists, bio-ethicists, politicians and the public, focusing on the use of human blastocysts for research. Now is a good time for scientists carefully to consider and communicate to the public the benefits of allowing such research, and to offer guidance on how it could be regulated to prevent ethical breaches. ■

Welcome SciDev.Net

This week sees the launch of an independent website for the developing world.

The exclusion of large parts of the world's population from many of the benefits of science and technology is a critical issue in international affairs. We are therefore pleased to announce our support for a new independent website, *SciDev.Net*, launched this week, which will provide a forum for authoritative news, information and comment about how science and technology can help meet the needs of developing nations. Although the site has been in gestation for more than a year, its appearance now could hardly be more timely. The range of potential topics to be addressed is broad, from global warming to genetically modified crops. The spirit is one of dialogue: it is as important to transmit perspectives held in the developing world as it is to convey those of 'the North'.

We have been closely involved in the incubation of *SciDev.Net* and (together with the journal *Science*) will be providing free access each

week to selected articles from our pages. Support and guidance is being given by the Third World Academy of Sciences, and funding generously provided by the UK Department for International Development, the Swedish International Development Cooperation Agency and the International Development Research Centre in Canada.

We hope that the information and perspectives offered by *SciDev.Net* will both strengthen the hands of policy-makers and empower individuals and communities, leading to sounder decision-making at all levels of society. By doing so, the website aims to help bridge the gap between the 'haves' and 'have-nots' in science and technology in development. We invite you to show your support by visiting the website (www.scidev.net), registering your interest, and engaging in the debates that we hope it will stimulate. ■

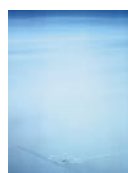




On the horizon
A non-NASA lab
leads New Horizons
mission to Pluto
p571



Culture shock
EU reluctant to pay
for world-beating
conservation work
p572



Deep freeze
Scientists plan to
explore Lake Vostok,
beneath Antarctica
p573



Art for the birds
Pigeons can learn
to distinguish Van
Gogh from Chagall
p574

Gene therapy may be up to speed for cheats at 2008 Olympics

David Adam, London

Athletes may be tempted to genetically modify themselves to boost their performance far sooner than most people realize, researchers have warned.

The idea of sprinters and cyclists injecting themselves with genes coding for hormones that boost the number of red blood cells may sound far-fetched, and the results are uncertain. But some gene-therapy researchers and sports organizations believe that such a genetically modified athlete could mount the winner's podium at the 2008 Olympic Games in Beijing.

If such 'gene doping' did take place, it could be impossible to detect, warns Peter Schjerling, a molecular biologist at the Copenhagen Muscle Research Centre. Speaking at a conference on genes and sport at University College London on 30 November, Schjerling said that artificial genes "can and most likely will be abused by healthy athletes as a means of doping".

Schjerling is not the first to voice concern that unscrupulous athletes might exploit emerging gene-therapy techniques currently being developed to treat conditions such as kidney failure and anaemia. The International Olympic Committee has set up an advisory group on the matter, and says it is



Where's the dope? A Swiss poster backs the International Olympic Committee's stand against drugs.

closely monitoring progress in gene therapy.

The World Anti-Doping Agency (WADA), a body coordinating a global campaign against the use of banned drugs in sport, is so concerned that it will hold a special conference to discuss the topic in March. (The meeting, at Cold Spring Harbor

in New York, was originally scheduled for September, but was postponed after the terrorist attacks on the United States.)

Schjerling says that athletes could target performance-enhancing genes such as those encoding growth factors capable of building muscle strength or widening blood vessels,

Poor practices led to BSE brains mix-up, say auditors

Declan Butler

Auditors have blamed Britain's Institute of Animal Health (IAH) for poor labelling and record-keeping practices that allegedly led to years of BSE research being wasted.

The Department of Environment, Food and Rural Affairs commissioned two audits last month, after tests by the Laboratory of the Government Chemist revealed that samples from brains being studied for signs of BSE in sheep had actually come from cattle (see *Nature* 413, 760; 2001). This triggered a controversy over whether IAH researchers had spent five years accidentally testing the wrong brains.

But the institute's director, Chris Bostock, rejects the auditors' reports and denies that a mix-up occurred at the IAH.

An audit by the UK Accreditation Service (UKAS), based in Newbury, Berkshire, was made public on 30 November. It found that the IAH's record-keeping system fell short of accepted good practice, that "there was no formal documented quality system" and that this "was inadequate to give confidence in the chain of custody of the samples".

The London-based auditors Risk Solutions said that poor labelling could have allowed a mix up of the original 1990 sheep tissue samples with bovine samples

that had been stored in the same freezer.

In a strong rebuttal, the institute said that it had in place "comprehensive and documented formal procedures" that met government standards.

Bostock says the audits were carried out hastily, and failed to inspect the institute's overall record-keeping system. "The audits fail to convince me that a mix-up occurred," says Bostock. The IAH is now completing an internal audit of the affair.

"It's time to move on," he says, adding that researchers should concentrate on the critical question of whether BSE is already in sheep flocks (see pages 576–577).

or a hormone called erythropoietin (EPO) that raises the number of oxygen-carrying red blood cells.

EPO abuse is already thought to be rife in many sports, including cycling — a whole team was expelled from the 1998 Tour de France for using it — but tests have been developed that can find synthetic versions of the hormone injected into the body (see *Nature* **407**, 124; 2000). Introducing the gene coding for EPO would circumvent the tests, however, because the extra hormone produced would effectively be endogenous.

The artificial gene could be delivered to the body in several different ways, experts say. Perhaps the easiest method would be to inject the DNA directly into the muscle. But more efficient techniques using viruses or modified cells from the patient are also being developed.

One problem that currently prevents the clinical use of these techniques is the difficulty of controlling which tissues receive the gene. Schjerling says this would be of little concern to an athlete who just wants a short-term boost in hormone levels. Equally, doubts about whether the genes would keep functioning over time would not concern someone preparing for a one-off event such as the Olympics.

Gene doping of this sort would be difficult to expose. "The DNA of the artificial gene itself can be detected," says Schjerling, "but this requires that the sequence be known and that a sample of the tissue containing it can be acquired."

Even if the risk of detection was small, the risk to the athlete's health probably would not be. This is where the almost fanatical desire of athletes to excel raises problems, according to some of those present at the London meeting. Several cyclists are already thought to have died from EPO use, as the marked increase in circulating red blood cells severely thickens the blood, increasing the risk of clots and strokes.

In animal studies undertaken at the Chiron Corporation in Emeryville, California (S. Zhou, J. E. Murphy, J. A. Escobedo & V. J. Dwarki, *Gene Therapy* **5**, 665–670; 1998), the levels of red blood cells in baboons given experimental EPO gene therapy rose so sharply (from 40 to 75% in 10 weeks) that their blood had to be regularly diluted to keep them alive.

Theodore Friedmann, director of the gene-therapy programme at the University of California, San Diego, and a member of the WADA health, medical and research committee, says gene doping may occur sooner than people think. "The technology needed for a rogue attempt won't take that long to develop," he says. Although he thinks it unlikely that any athlete has yet tried it, he adds: "We all think there will be an attempt to do so."

Cell biologist quits editorial board over cloning paper

Jonathan Knight, San Francisco

Stem-cell pioneer John Gearhart has resigned from the editorial board of the biomedical journal that last month published a controversial paper claiming the creation of the first cloned human embryos.

Gearhart, a biologist at Johns Hopkins University in Baltimore, Maryland, says that in agreeing to publish the work, the journal had failed to uphold scientific standards.

The article appeared in the 26 November issue of the online journal *e-biomed: The Journal of Regenerative Medicine* (**2**, 25–31; 2001), published by New York-based Liebert.

Using the technique that created Dolly the sheep, the researchers, led by Jose Cibelli and Michael West of Advanced Cell Technology, a biotechnology firm in Worcester, Massachusetts, transferred nuclei from human cumulus cells, which surround eggs after ovulation, to human eggs (see *Nature* **414**, 477; 2001). A few of the eggs divided once or twice before they died.

The company claimed that it had finally cloned humans, adding that it had achieved

an important first step in developing a technology that will ultimately revolutionize medicine.

But Gearhart says: "I don't think they have come anywhere near the mark of what it would take to prove that claim." He argues that the research is lacking in several important respects, such as its failure to provide evidence that the DNA in the dividing eggs actually came from the donor cell, or that it was functional.

Gearhart says he sought an explanation from the journal's editor-in-chief William Haseltine — chairman of Human Genome Sciences, a Maryland-based biotechnology company — as to how the paper passed editorial muster, but that none was forthcoming. He says he informed Haseltine of his decision to resign in an e-mail on 3 December.

Haseltine defends the decision to publish the paper. "Our reviewers thought it was an advance over what had been done before," he says. "It is not the ultimate word in embryogenesis, but it has not been described as that."

Anthrax evidence implies US culprit

David Adam

The investigation into the anthrax attacks in the United States may be homing in on the unsettling prospect that a researcher from America's own biological weapons programme is responsible.



Home-made? Those behind the anthrax spores sent to Congress may have had access to US labs.

Barbara Rosenberg, a microbiologist at the State University of New York who chairs the biological weapons working group at the Federation of American Scientists, claimed this week that the investigations point to a microbiologist who once had access to weaponized anthrax in a US government lab. "I think the FBI knows it and I think they're looking into it," she says.

Rosenberg voiced her concerns at the Biological Weapons Convention meeting in Geneva on 21 November and then posted them on a listserv hosted by the Stockholm International Peace Research Institute.

The FBI has already indicated that it believes the anthrax culprit is probably based in the United States and recently confirmed that it is investigating US labs capable of producing the high-quality powdery form of the bacteria used in the attacks.

Rosenberg argues, among other things, that tests on the anthrax samples have revealed the presence of a chemical known to have been used in the US process that turns wet slurries of anthrax culture into spores suitable for weapons.

Other scientists involved in bioweapons research have reacted more cautiously. "The general outline has some credence but it is by no means certain," one said.

Wandering Chinese fossil turns up at museum

Rex Dalton, San Diego

A controversial Chinese dinosaur fossil, which has meandered for years through the international underground world of dealers, has been bought by a leading German museum, the institution's director said last week.

The psittacosaurid fossil — with a tail clad in spines reminiscent of the quills of a porcupine, never before seen on such specimens — is now at the Naturmuseum Senckenberg in Frankfurt, acknowledged palaeontologist Fritz Steininger. The specimen was purchased this summer from a German dealer, Ulrich Leonhardt, for around US\$200,000, which is being paid in instalments, said Steininger.

Some prominent palaeontologists believe that the fossil was smuggled out of China, and the propriety of its purchase by the Senckenberg is expected to stir intense debate in the palaeontological community.

Many major museums would have nothing to do with such a specimen, because their rules prevent them from handling fossils of questionable provenance. For instance, the American Museum of Natural History in New York wouldn't allow the specimen in the door, says palaeontology curator Mark Norell. Norell, who works extensively in Asia, says the fossil has been "stolen from China".

Steininger took issue with such an assessment. He says his museum has proper German importation documents and exportation records from the United States, where he said Leonhardt bought it. But Steininger would not discuss any authenticating papers from China.



"We have requests from people to come and work on the specimen," says Steininger. "Everyone who is entitled to can study it. We are trying to do our best."

The museum is reconstructing an almost-complete fossil of the animal, which was at least a metre long and lived more than 100 million years ago. The quills on its tail seem to be different from the feather-like integument found in other dinosaurs. The existence of a dinosaur with such unusual integumentary structures will provoke considerable interest among palaeontologists.

But Senckenberg scientists may find it difficult to publish their results in a reputable journal, given the fossil's background. Most journals require records documenting a specimen, such as a museum number. Historically, peer reviewers for leading journals have recommended rejecting manuscripts on specimens lacking such a number.

Steininger revealed that the fossil was at his museum after learning that *Nature* was preparing an article on its globe-trotting trail into the hands of the dealer, whose firm, Leonhardt & Partner, is based in Kirchheim, Germany. Two years ago, before it was bought by the Senckenberg, Leonhardt allowed an Italian dealer to take the specimen to the Museo Civico di Storia Naturale in Milan, Italy, for analysis (see *Nature* 412, 844; 2001).

The dealer, Flavio Bacchia of the Stone Age shop in Trieste, Italy, said Leonhardt returned to Germany with the specimen after Milan scientists learned of the fossil's significance. Earlier this year, a palaeontologist spotted the crated fossil in a vehicle in a parking lot near the University of Tübingen in Germany, being offered for sale for \$300,000. Leonhardt declined to be interviewed.

Previously, the specimen had made its way around the world, first surfacing some three years ago at a fossil and mineral show in Tucson, Arizona. A dealer there sold it to an American museum, which later returned it. The dealer then sold it to Leonhardt, said Steininger.

When the specimen was in Milan last year, it was examined by palaeontologist Eric Buffetaut of the CNRS, France's national research agency. Realizing its importance, Buffetaut tried unsuccessfully to organize its repatriation to China. A retired Chinese palaeontologist from Beijing's Institute of Vertebrate Paleontology and Paleoanthropology visited Milan to negotiate its return, but failed to do so (see *Nature* 414, 147; 2001).

NASA engages outside help for mission to Pluto

Tony Reichhardt, Washington

For the first time ever, NASA has asked an outside laboratory to lead a reconnaissance mission to an unexplored planet.

The space agency earlier this year funded two feasibility studies for a mission to Pluto.

And on 29 November it announced that it had chosen for further development the proposal from a team led by Alan Stern, a planetary scientist at the Southwest Research Institute in Boulder, Colorado. Called 'New Horizons', the mission could launch in January 2006.

The spacecraft, to be built and operated by the Applied Physics Laboratory at Johns Hopkins University in Baltimore, would examine Pluto and its moon Charon. It would then go on to explore other small bodies within the Kuiper Belt.

But to survive beyond next year, the mission must pass a NASA technical and fiscal review, and secure long-term funding from Congress. The White House requested no funds for a Pluto mission in 2002, and the project went ahead only after a last-minute

congressional appropriation of \$30 million.

If it launches in 2006, the spacecraft will reach Pluto between 2014 and 2018, depending on the launch vehicle selected. On board will be equipment to characterize the geology and map the surface composition of Pluto and Charon. The planet's atmosphere will also be assessed.

New Horizons beat the mission proposal that would have been managed by the Jet Propulsion Laboratory (JPL) in Pasadena, California, which usually runs NASA's planetary science missions. Originally, NASA had wanted the JPL to develop both the Pluto mission and a mission to Jupiter's moon Europa. But when the costs for the Europa orbiter mushroomed, the two projects were 'decoupled', and the Pluto mission was opened to outside competition.



Fresh vistas: the Pluto mission will go on to probe other small bodies in the Kuiper Belt.

European Parliament rejects move to restrict genetics

Quirin Schiermeier, Munich

The European Parliament has ditched a report from one of its committees that would have called for tighter restrictions on genetics and biotechnology.

The action was welcomed by researchers and biotechnology companies, who opposed the report's recommendations and were concerned that the parliament might use its growing influence to restrain their work.

The report, prepared by a multiparty committee on human genetics and modern medicine, would have called for a ban on the cloning of human embryos for any purpose, and an end to the granting of gene patents. But on 29 November, the full parliament rejected the report by 316 votes to 37.

The 72-member committee's final report, produced after a year of expert hearings, was "very negative for science", admits Robert Goebbels, a Social Democrat from Luxemburg. Goebbels chaired the committee and was obliged to bring the report to the full parliament, even though he disagreed with it.

Last-minute attempts to make the report more pro-science failed to rescue it. In a turbulent session last Thursday, the parliament adopted 230 amendments introduced by various groups. But the result was riddled with contradictions and, says Goebbels, was "simply not good enough to be supported".

"It was not an hour of glory for the parliament," he says. But he notes that scientists will be relieved at the report's demise, and says that it's better to have no report than a bad report. "I am not happy that we spent 11 months without getting a decent result," he adds. "But there has been an evolution in the minds of many colleagues, who are now much better acquainted with the problems of modern biotechnology."

Conservatives were also unhappy at the report's failure. "It is a shame that no clear view could be adopted," says Peter Liese, a German Christian Democrat who strongly opposes cloning and human embryonic stem-cell research.

The European Parliament has already set out its stall on some biomedical research issues in its proposal for the next European Union (EU) Framework programme for research. That document said that EU funding should be used for stem-cell research, but that no human embryos should be cloned for research purposes (see *Nature* 414, 386; 2001). ■

Rescuers of Europe's cultural heritage struggle for funding



Unalloyed success: new shape-memory alloys helped rebuild the Basilica of St Francis in Assisi.

Alison Abbott, Munich

Scientists who work to conserve historic buildings, monuments and archaeological remains face an uphill struggle to secure continued funding from the European Union — despite their considerable success.

Research on the conservation of cultural heritage, which has been funded for the past 15 years by the European Commission, has been an outstanding model of effective technology transfer, says a report. The study, which was prepared by the European Parliament's Scientific and Technological Options Assessment unit (STOA), adds that Europe leads the world in this research area.

But the report notes that such research is likely to be excluded from the European Union's next Framework research programme (FP6), which starts in 2003.

May Cassar, director of the Centre for Historic Buildings, Collections and Sites at University College London, who helped to prepare the report, says that FP6's emphasis on large grants of up to 10 million euros (US\$8.9 million) will exclude cultural heritage networks, which require more modest funding. "It is crazy to abandon research at this point when it is starting to bear fruit, and when we are the envy of our colleagues in Asia and the Americas," she says.

The report's authors ask the commission to add a special funding line to FP6 for developing technological solutions for the conservation and protection of cultural heritage. There are no other sources of international funding for this work, they point out.

The FP6 is not yet set in stone — its final shape will be agreed next year by the commission, the parliament and the European Council, which is made up of representatives of the European Union's member govern-

ments. Although the parliament has proposed amendments that would include cultural heritage in FP6, the commission is not in favour of the change.

A spokeswoman for research commissioner Philippe Busquin says the present text of the Framework programme is "not a vote against cultural heritage". That cannot be a priority, she says, "but it will find its place in other specific FP6 programmes such as nanotechnology where new materials may prove advantageous for restoration work."

Since the European Union began funding research into the protection of cultural heritage, many different problems have been approached by interdisciplinary teams, often making use of materials and techniques originally developed for other purposes.

One project adapted a 'shape-memory alloy', a highly elastic nickel-titanium alloy used in industrial and dentistry applications, to stabilize the structure of historic monuments in earthquake-prone areas. The project, begun in 1996, proved timely: the alloys were used in the reconstruction of the Basilica of St Francis in Assisi, Italy, after it was badly damaged by an earthquake in 1997.

Technology transfer has worked in both directions, says Cristina Sabbioni, an atmospheric physicist at the CNR Institute for Atmospheric and Oceanic Science in Bologna and a STOA committee member. For example, a new species of antibiotic-producing bacterium was discovered by an international team looking at the effects of mass tourism on prehistoric rock art in the Altamira caves in northern Spain. The Hans Knöll Institute for Natural Products in Jena, Germany, has patented the novel antibiotic, which it has named altamiramycin. ■

♦ www.europarl.eu.int/stoa

Researchers plan probe into Antarctic lakes

Helen Gavaghan, Bologna

The subglacial lakes of east Antarctica — the last great unexplored region on Earth — could soon yield their secrets to science. An international group of researchers met in Bologna, Italy, last week to draft plans for a coordinated study of the region.

The Antarctic lakes lie beneath ice 4 km deep, and there are concerns that drilling into it might contaminate an environment that has been isolated from the rest of the world for millions of years. The Group of Specialists on Subglacial Antarctic Lake Exploration (GOS-SALE), which held the meeting, knows that its biggest challenge may be to convince conservationists that the project won't damage the environment.

Studying the lakes might resolve some major questions about the Earth's geological history, according to group member Ross Powell, a geologist at Northern Illinois University. And biologists are excited by the chance to study life forms from the isolated, subglacial ecosystem, says John Priscu of Montana State University, the group's chair.

The study might cost tens of millions of dollars, however, and the proposal is far from being accepted either by scientists or their political masters. Yet the presence in Bologna of key officials, such as Karl Erb, director of polar programmes at the US National Science Foundation, suggests that it has a good chance of coming to fruition.

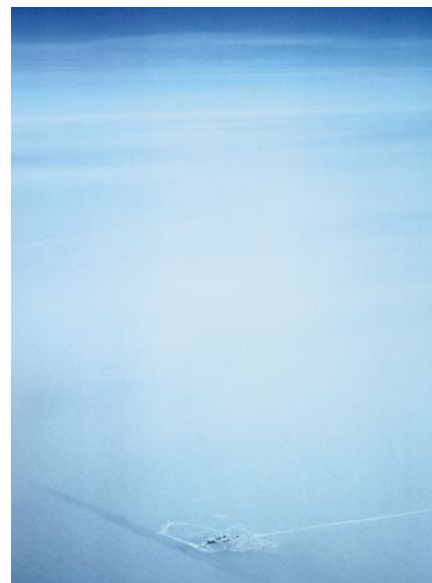
Valery Lukin of the Russian Arctic and

Antarctic Research Institute used the forum to suggest extending an existing, 3.6-km-deep borehole over the largest lake, Lake Vostok, that is already used to survey the Earth's climate history. Lukin invited international collaboration in the extension project, which would take samples of frozen water from above the lake but beneath the ice cap.

Recent data raise intriguing questions for further study. For example, Robin Bell, a geophysicist at the Lamont-Doherty Earth Observatory at Columbia University in New York, presented findings from a \$5-million survey of Lake Vostok, showing that the flow of ice across the lake is different from what had been anticipated. Bell also believes that the edge of the Antarctic continental shelf runs beneath Lake Vostok, rather than past the continent's western edge, as had been assumed.

Sergei Bulat of the St Petersburg Nuclear Physics Institute and Priscu presented DNA and microbial data, respectively, suggesting that life in Lake Vostok is widely dispersed, and may not be easily found by drilling into a single location.

While the US and Russian researchers concentrated on Lake Vostok, Italian scientists have studied the region around Concordia, a Franco-Italian base 600 km to the south. Ignazio Tabacco, a geophysicist at the University of Milan, provided evidence of a second large lake, and suggested that a string of other lakes in the area may be linked. The



Ice house: the isolated Vostok station monitors the vast lake system underneath Antarctica's ice.

ultimate goal is to take samples from Lake Vostok, but there is some support for a plan to explore one of the other lakes first.

The meeting did not reach a firm plan, but officials believe that one will take shape soon. GOS-SALE intends to submit a research plan in July 2002 for endorsement by its parent body, the Scientific Committee on Antarctic Research. Individual nations will then determine whether or not to participate. ■

France boosts funding for biotechnology start-ups

Sally Goodman, Paris

France has announced a plan to bolster its lagging biotechnology sector by injecting fresh public money into private biotech companies.

Presenting details of the plan to parliament last week, finance minister Laurent Fabius said that the government will provide 60 million euros (US\$54 million) next year for new biotechnology start-ups, and an additional 90 million euros in loan guarantees for more established companies.

Fabius hopes that the money, provided as part of the 2002 budget outlined by the Ministry of Finance in October, will be supplemented by the European Investment Bank. It will be invested in ventures that have already obtained some initial funding.

The government hopes that its actions will enable France to emulate Germany, which has doubled public investment in biotechnology over the past five years and taken over from Britain as Europe's leader in private biotechnology investment (see graph). France

says it wants to overtake Germany by 2006.

"The announcement has generated a lot of enthusiasm in the industry," says Philippe Pouletty, president of France Biotech, an industry association that has been lobbying the government over what it regards as under-investment in biotechnology.

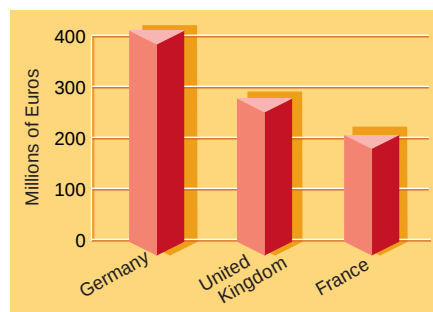
"The 2006 objective is realistic," says Thierry Laurent, an economist from the

University of Évry-Val d'Essonne near Paris. But he says that other sources of investment, such as the national health insurance fund, need to be tapped if the objective is to be met.

The government hopes that the 90 million euros in guaranteed loans will leverage up to 450 million euros of private investment in French biotechnology companies.

Publicly funded research in life sciences, which receives a quarter of France's total research and development budget, stands to benefit indirectly through more technology transfer with the biotechnology companies. "This is an exceptional opportunity for the public sector," says Bernard Pau, director of the biotechnology institute at the University of Montpellier.

The number of start-ups founded by academic researchers in all disciplines in France has increased fivefold since 1999, when a law was passed allowing publicly supported researchers to become shareholders in companies linked to their laboratories (see *Nature* 397, 187; 1999). ■



Mind the gap: in 2000, France lagged behind its rivals in private-sector biotech investment.

Russia offers star prize in bid to boost space funds

Moscow Funding mechanisms for the Russian space programme moved from the sublime to the ridiculous this week with the announcement that a television game show offering prizes of two seats on board a Soyuz rocket, and a possible visit to the International Space Station (ISS), will be used to generate revenue.

Thanks to an agreement between MirCorp, a space tourism company based in Amsterdam, the Russian Space Agency and Russian rocket manufacturer RSC Energia, two winners of the 'Ancient Astronaut' show will go on missions lasting 8–10 days

onboard a Soyuz rocket in 2003.

MirCorp says the trips may include a visit to the ISS, but agreement has not been reached with either NASA or the European Space Agency. NASA objected earlier this year when Russia sold a seat on a Soyuz mission to the ISS to American millionaire Dennis Tito. The agency was eventually forced to allow Tito onboard the ISS, and may again be unable to prevent Russia using its allocated slots on the station as it sees fit.

German ethics council backs stem-cell plans

Munich Germany's lengthy debate over the use of human embryonic stem (ES) cells took another twist last week when the high-level National Ethics Council backed the use of imported ES cells in research, with a review of the situation to come after three years.

The creation and use of human embryos for research purposes is banned in Germany, but research on imported ES cells is legal. Germany's main university granting agency, the DFG, wants to fund research involving imported ES cells, but intense public and political debate over the issue has forced it to delay a ruling on the matter.

The decision by the ethics council, which was established last year by Chancellor Gerhard Schröder and consists of academics

from the arts, sciences and humanities, will bolster the DFG's position. The agency is scheduled to rule this month, but is under growing pressure to postpone its decision until after the German parliament's debate on the use of ES cells, scheduled for January.

End in sight for cloned bull's chaste existence

Milan Galileo, the Italian cloned bull, is likely to have his enforced celibacy revoked at some point during the new year.

Galileo was born in January 1999 at the Laboratory of Reproductive Technology, part of animal biotechnology company CIZ, based in Cremona, Lombardy.

The bull was produced as part of a project aimed at improving the quality of cattle stocks, but he was impounded in September 1999 following a ban on animal cloning that was introduced by former health minister Rosy Bindi.

A court ruling secured Galileo's freedom in February this year, but the laboratory was instructed not to take any semen from him while the ban remained in place. According to Italian news reports, the current health minister, Girolamo Sirchia, is now intending to lift the ban next year.

Cesare Galli, head of the CIZ laboratory, says that his scientists are poised to move

AP



What a blast: game-show winners could take a trip into space on Russia's Soyuz launch rocket.

ahead rapidly with their animal cloning research, which he says they had not abandoned during the temporary ban.

White House praises NSF but warns of belt-tightening

Washington The outlook for the National Science Foundation (NSF) under the Bush administration brightened last week, when a top White House official named the agency as one of three “true centres of excellence” in the federal government.

Mitch Daniels, director of the powerful Office of Management and Budget, said in a talk to Washington’s National Press Club that more than 95% of funds provided to the NSF “go out on a competitive basis directly to researchers pursuing the frontiers of science, a very low overhead cost”. NSF officials hope that these comments indicate that the administration may look favourably on their pleas for more funding.

Daniels also praised the National Weather Service and a Department of Agriculture nutrition programme for women and children.

He went on, however, to warn of a coming era of government belt-tightening. War spending and a slowing economy will make it essential to “separate ‘must-do’ from ‘nice-to-do’ items”, he said.



Bird brain? Pigeons learnt to identify Van Gogh paintings after seeing works such as *Sunflowers*.

Art-critic course puts pigeons in the picture

London Pigeons can tell their Van Gogh from their Chagall, a new study has shown.

The birds took an art appreciation course in which they were rewarded for choosing paintings by Van Gogh but discouraged from choosing Chagalls. After the initial viewing, which included works from Van Gogh’s famous sunflower series, the avian art critics

were challenged to distinguish between previously unseen works by the two painters.

Telling Dutch impressionist from French modernist proved easy work for the pigeons. The fastest bird pecked preferentially at Van Gogh’s work after just nine classes, and the pigeons as a whole performed almost as well as a parallel group of humans (S. Watanabe *Animal Cognition* **4**, 147–151; 2001).

Harvard offers reward for news of missing biologist

San Diego A US\$10,000 reward has been offered for information about the disappearance of structural biologist Don Wiley. The money was put up by Harvard University, where Wiley was based, and the St Jude Children’s Research Hospital in Memphis, Tennessee, where he was last seen leaving a dinner after a scientific board meeting on 15 November.

Wiley’s rental car was found outside Memphis early the next day on a bridge over the Mississippi River. His family say they do not believe he killed himself. But Memphis police say it looks like suicide and they have found no evidence of foul play. Reports that the Federal Bureau of Investigation are involved have been denied, and blamed on ambiguous information supplied to reporters by a local police officer.

A wolf in sheep's clothing

Is BSE lurking in sheep, but masked by scrapie? Reliable and fast tests that can tell the diseases apart are urgently needed, says Declan Butler.

After years of research, we're still no closer to knowing whether the epidemic of bovine spongiform encephalopathy (BSE) that ripped through British cattle herds from the mid-1980s also spread to sheep. In October, researchers were poised to reveal that they had found the tell-tale signature of the BSE agent in a sample of sheep brains dating from the early 1990s, when a last-minute government statement threw their work into confusion. The tests, it was announced, had been conducted in error on cattle brains, because of a mix-up of samples¹.

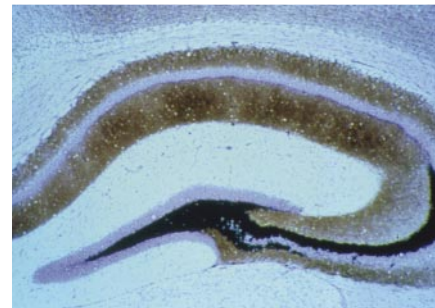
But while the lamb industry is off the hook for the time being, the risk that BSE is lurking in sheep flocks is real, as sheep were fed the same contaminated feed blamed for the disease's spread through cattle. If so, it would be impossible to distinguish the symptoms from scrapie, a naturally occurring disease of sheep.

That is a worrying proposition. Experiments in which sheep have been infected with BSE indicate that a greater proportion of tissues become infective than in a cow with BSE² — meaning that lamb might even have to be pulled off the market altogether. And unlike BSE in cows, which should be eradicated in a matter of years now that contaminated feed is not in use, scrapie passes readily from sheep to sheep in ways that are not fully understood. If BSE in sheep behaved similarly, it might not go away. The British government's contingency plans, released for consultation in September, reflect these worries — even suggesting that the entire national flock of up to 40 million animals might have to be slaughtered.

If nothing else, the 'wrong brains' fiasco revealed the importance of developing a fast



P. DEAN/AGRIPICTURE



J. FRASER/EURELIOS/SPL

Scrapie in a slice of sheep brain (left) showing loss of grey matter compared with a healthy brain.

diagnostic test that can distinguish BSE from scrapie. The researchers had tested a pooled sample of brains by injecting the liquidized tissue into different strains of mice and looking at the disease incubation times and the pattern of the brain lesions that formed. This method, developed by Moira Bruce of the Institute for Animal Health (IAH) in Edinburgh, provides a reliable way of distinguishing between different strains of scrapie, and of identifying the BSE agent. But with read-out times of up to two years, it can hardly be described as fast.

Prion probes

No rapid diagnostic test has yet been validated for detecting transmissible spongiform encephalopathies in sheep, let alone one that can distinguish between BSE and scrapie. In both diseases, a cell-membrane protein called PrP, which consists mainly of conformations called α -helices, is converted to the abnormal 'prion' form, PrP^{Sc}, which consists mainly of β -sheets (see figure, opposite). Three rapid tests, which use antibodies to detect the rogue form, are reliable detectors of BSE in cows showing symptoms, and have been approved for use in cattle by the European Commission³.

These tests — marketed by Prionics of Zurich, Enfer Scientific of Tipperary, Ireland, and Bio-Rad of Hercules, California — have also been put through preliminary trials on sheep with scrapie, and seem to perform well, according to European Commission officials. Applying them to sheep would be useful, as almost nothing is known about the epidemiology of scrapie in Europe. And if used in abattoirs, they could help keep infected sheep out of the food chain.

But to get to the bottom of the issue, we need a test that can distinguish between the two diseases. The best-known candidate was developed by John Collinge of Imperial College, London. His test involves loading samples of PrP^{Sc} onto a gel, using electrophoresis to separate the protein into different bands and then performing a standard western blot procedure to identify the rogue prion. In 1996, he showed that PrP^{Sc} from human patients with vCJD, the variant of Creutzfeldt-Jakob disease apparently caused by the BSE agent, forms a distinctive pattern of bands that distinguishes it from other forms of CJD⁴. The differences are thought to arise from variations in the sugar groups attached to the protein.

The method is cheap and, in experienced

hands, hundreds of samples can be processed in 48 hours. But to obtain good results, large quantities of protein must be loaded onto the gels, which can make the test difficult to perform. "You need something that works in everyone's hands 100% of the time," notes one prion researcher.

Nevertheless, scientists at the UK Central Veterinary Laboratory (CVL) in Weybridge, Surrey — part of the Veterinary Laboratories Agency (VLA) — have been trying to determine whether a western blot procedure can also identify BSE in sheep. Collinge has publicly criticized the Department for Environment, Food & Rural Affairs (DEFRA), which is sponsoring the research at the VLA, for the time it has taken to adapt the test for use in sheep.

Digestive aids

The VLA is working on a number of tests that might be able to distinguish BSE from scrapie. It is modifying the Prionics test to incorporate a variant of Collinge's method. In addition, Michael Stack, a researcher at the CVL, has identified a 'signature' at one end of the fragment that remains when PrP^{Sc} is digested with a protease enzyme. The point at which the enzyme cleaves the protein seems to vary depending on whether the prion is BSE or scrapie, and antibodies can target this difference. The CVL is now collaborating with Prionics to turn this into a commercial test.

Another method, developed by Martin Jeffrey's group at the VLA in Lasswade, near Edinburgh, uses two different antibodies to stain samples of brain and lymphoid tissue, giving patterns for scrapie and for BSE that Jeffrey argues can be distinguished by a trained eye.

Last month, David King, the UK government's chief scientific adviser, announced plans to screen 20,000 sheep for prion disease, using the basic Prionics test and a test related to the one developed by Jeffrey that does not distinguish between BSE and scrapie. Positive results will be further investigated using the VLA's prototype tests to look for suspicious BSE-like signals.

In the meantime, other groups are also trying to devise tests that could dis-

tinguish BSE in sheep. Enfer Scientific is working with an antibody that it believes is specific for scrapie PrP^{Sc}. Samples that test positive on its current test but negative using this antibody would be BSE, says Riona Sayers, who coordinates the company's research. The technique discriminates cattle with BSE from sheep with scrapie, she says.

The researchers at the French Atomic Energy Commission (CEA) who developed the test sold by Bio-Rad are also working on a two-step test, but one which exploits the fact that scrapie PrP^{Sc} from sheep is more resistant than BSE PrP^{Sc} from cattle to digestion by an enzyme called proteinase K. They assume that the BSE prion in sheep will be similarly susceptible to the enzyme. Samples testing positive using the existing Bio-Rad test are retested after applying a concentration of proteinase K that obliterates all BSE PrP^{Sc}, but leaves scrapie PrP^{Sc} detectable. In cows with BSE, the signal disappears, whereas in sheep with scrapie it persists. Preliminary tests on 300 sheep showing symptoms of scrapie have shown a handful of BSE-like responses.

Unfolding issues

Other researchers have different approaches. Jiri Safar, working in the lab of Stanley Prusiner at the University of California, San Francisco, is developing a test that depends on the way in which different versions of PrP^{Sc} unfold when they are treated with a chemical called guanidine hydrochloride⁵. Meanwhile, Alex Bossers and his colleagues at the Institute for Animal Science and Health in Lelystad, the Netherlands, are working from the observation that PrP^{Sc} converts PrP to its own prion form more quickly if it comes from the same species. BSE in sheep might therefore show different conversion rates from scrapie, and it should be possible to measure this.

Given that any test can be subject to false positives and false negatives, and the high stakes involved, experts say it will be important to use several tests in parallel to cross-check results — as the British programme will do. But Safar is worried that none of the tests is yet sufficiently well developed to be



Head start: John Collinge's test might identify BSE in sheep.

relied upon. Applying them too quickly, he says, "is a very dangerous strategy that risks generating a lot of controversial findings which cannot be verified in a reasonable time".

The main immediate bottleneck for those developing diagnostics is getting sufficient BSE-infected sheep tissues — most of which are held by the CVL — to validate the prototype tests. Jacques Grassi, a member of the CEA team, says that its new test works well on samples of mice infected with either BSE or scrapie. But so far the team has been able to try it on just five sheep infected experimentally with BSE. "We need more tissues," he says.

Aside from the reliability of the nascent diagnostic technology, the main theoretical problem is our ignorance of the variety of scrapie strains circulating in the field, and of the behaviour of these strains — and of BSE — in sheep of different genotypes. "These two areas are the major limiting factors," says Stephen Edwards, chief executive of the VLA.

So far, for instance, only one breed of sheep has been deliberately infected with BSE. The CVL has begun a new series of experiments to generate infected tissues from other breeds, but these tissues will not become available for another two years. Until these samples can be examined, there is a danger that using the tests in the field might give false positives — or false negatives — in particular breeds of sheep.

BSE seems to behave as a single strain, which is unchanged on transmission to other species. But few strain-typing tests have been done, and experiments with scrapie in rodents have shown that the characteristics of a strain can change if it is repeatedly transmitted from animal to animal⁶. "BSE might change when passed from sheep to sheep," says Bruce.

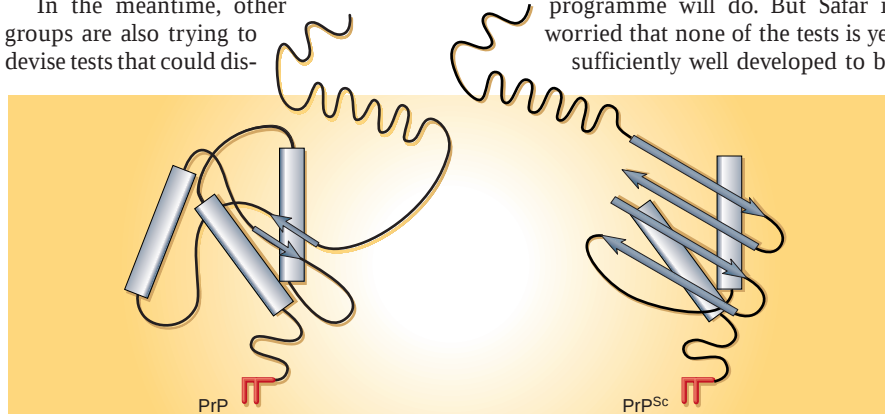
Given these complications, and the dire consequences of producing a positive test for BSE in sheep, the scientists developing the tests are under intense pressure. Weighing on their shoulders is the potential danger to public health — not to mention the livelihoods of thousands of farmers.

Declan Butler is Nature's European correspondent.

1. Butler, D. *Nature* **413**, 760 (2001).
2. Foster, J. D., Bruce, M., McConnell, I., Chree, A. & Fraser, H. *Vet. Rec.* **138**, 546–548 (1996).
3. Schiermeier, Q. *Nature* **409**, 658–659 (2001).
4. Collinge, J., Sidle, K. C. L., Meads, J., Ironside, J. & Hill, A. F. *Nature* **383**, 685–690 (1996).
5. Safar, J. et al. *Nature Med.* **4**, 1157–1165 (1998).
6. Kimberlin, R. H., Walker, C. A. & Fraser, H. *J. Gen. Virol.* **70**, 2017–2025 (1989).

UK government contingency plan

► www.defra.gov.uk/corporate/consult/sheepbse/index.htm



Deadly change: some α -helices (grey tubes) in PrP are refolded as β -sheets (blue arrows) in prions.



The budding amateurs

From the results of an annual Alaskan betting contest to sightings of migratory birds, ecologists are using a wealth of unusual data to predict the impact of climate change. John Whitfield rummages in the archives.

Tim Sparks slides a small leather-bound notebook out of an envelope. The book's yellowing pages contain beekeeping notes made between 1941 and 1969 by the late Walter Coates of Kilworth, Leicestershire. He adds it to his growing pile of local journals, birdwatchers' lists and gardening diaries. "We're uncovering about one major new record each month," he says, "I still get surprised."



Around two centuries before Coates, Robert Marsham, a landowner from Norfolk in the east of England, began recording the life cycles of plants and animals on his estate — when the first wood anemones flowered, the dates on which the oaks burst into leaf and the rooks began nesting. Successive Marshams continued compiling these notes for 211 years¹.

Taking note:
Robert Marsham.

Today, such records are being put to uses that their authors could not possibly have expected. These data sets, and others like them, are proving invaluable to ecologists interested in the timing of biological events, or phenology. By combining the records with climate data, researchers can reveal how, for example, changes in temperature affect the arrival of spring, allowing ecologists to make improved predictions about the impact of climate change.

A small band of researchers is combing through hundreds of years of records taken by thousands of amateur naturalists. And more systematic projects have also started up, producing an overwhelming response. "The amount of interest is almost frightening," says Sparks, a climate researcher at the Centre for Ecology and Hydrology in Monks Wood, Cambridgeshire.

Sparks first became aware of the army of

"closet phenologists", as he describes them, when a retiring colleague gave him the Marsham records. He now spends much of his time following leads from one historical dataset to another.

As news of his quest spreads, people tip him off to other historical records, and more amateur phenologists come out of their closets. The British devotion to recording and collecting makes his job easier — one man from Kent sent him 30 years' worth of kitchen calendars, on which he had noted the date that his neighbour's magnolia tree flowered.

Changing times

Other researchers have unearthed data from equally odd sources. Rafe Sagarin, an ecologist at Stanford University in California, recently studied records of a betting contest in which participants attempt to guess the exact time at which a specially erected wooden tripod will fall through the surface of a thawing river. The competition has taken place annually on the Tenana River in Alaska since 1917, and analysis of the results showed that the thaw now arrives five days earlier than it did when the contest began².

Overall, such records have helped to show that, compared with 20 years ago,

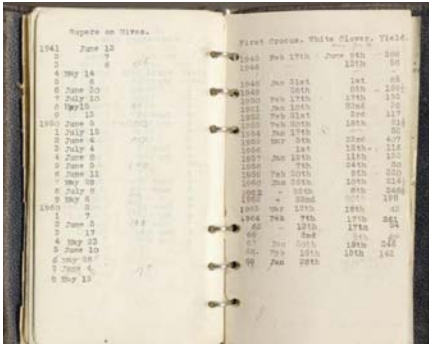
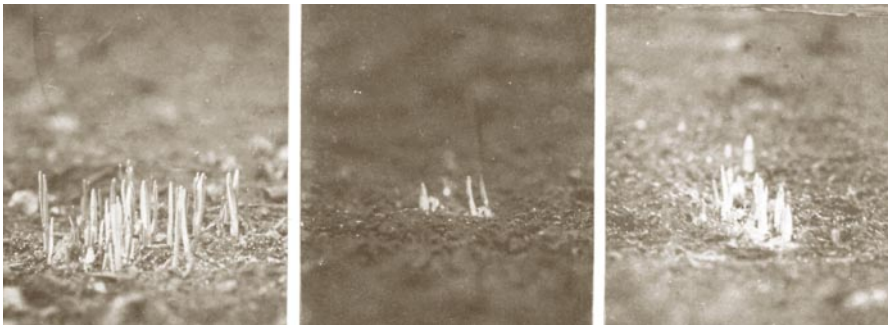
a raft of natural events now occur earlier across much of the northern hemisphere, from the opening of leaves to the return of birds from migration and the emergence of butterflies from hibernation. The data can also hint at how nature will change in the future. Hawthorn trees, for example, flower about 10 days earlier for every degree of warming, compared with between three and four days for hazel³.

Together with models of climate change, amateurs' records could help guide conservation. Terry Root, an ecologist at the University of Michigan in Ann Arbor, has collected birdwatchers' counts of wildfowl taken between 1955 and 1996 on seasonal ponds in the American Midwest and combined them with climate data and models of future warming. Her analysis shows that the increased droughts that the models predict could halve the breeding populations at the ponds⁴. "The number of waterfowl in North America will most probably drop significantly with global warming," she says.

But not all professionals are happy to use amateur data. "A lot of scientists won't touch them, they say they're too full of problems," says Root. Bias can arise in a variety of ways. "When the



Well spotted: large-scale surveys, such as on bird migration, rely on data collected by volunteers.



On the record: the same snowdrop clump captured every New Year's Day from 1922, and Walter Coates' beekeeping records (left).

process, causing them to fall earlier. He advocates a more involved approach than simply noting dates⁶, including measuring growth at the cellular level.

Plus, says Worrall, data for at least 50 years are needed before climate changes can be detected amid the natural variability. "I've been recording the daffodils in my backyard for 30 years, and I don't see any trend," he says. "The year-to-year variation is extraordinarily large: it's a mountain range." Sparks, in contrast, says that trends can emerge in as little as a decade in records of species that are very sensitive to temperature, such as hawthorn or some butterflies, and that 20 years is often a useful minimum.

Every note counts

Overall, most phenologists are positive about the contribution that amateurs can make. "They get at the raw power of science: careful observation of the natural world," says Sagarin. But the professionals also acknowledge the need for careful quality control. Root, for example, tries to gauge the quality of an amateur archive by interviewing its collector. "You always have to worry — things as trivial as vacations can affect measurement. I disregard a lot of records because they're not rigorous enough," she says.

Others suggest that the right statistics can iron out some of the problems with amateur data. Together with colleagues at Wageningen University in the Netherlands, environmental scientist Arnold van Vliet is developing statistical techniques to account for the uncertainty in amateur phenological data.

With the enthusiasm of amateur phenologists evident from past records, professional researchers are now trying to create standardized recording schemes for future efforts. They hope that well-designed studies will generate a volume of observations large enough to drown out the idiosyncrasies of individual recorders. The data are cheap to collect, and can provide breadth in space, time and range of species. "It's very difficult to collect data on a large geographical scale without enlisting an army of observers," says Root.

In collaboration with the Woodland Trust, a British conservation charity, the Centre for Ecology and Hydrology has created the Nature's Calendar project to coordinate amateur phenologists. Launched last spring, the network started with 300 observers. Thanks to publicity gained from *Today*, a British current-affairs radio programme, this autumn 10,000 volunteers recorded the last date on which they mowed their lawns, when leaves fell from trees, and the arrival of winter migrant birds.

The European Union is funding the European Phenology Network (EPN), based at Wageningen University, to promote cooperation between national phenological networks and to act as a clearing house for data. When the Dutch radio programme *Vroege Vogels (The Early Bird)* heard about the EPN it contacted van Vliet, the network's coordinator, and launched a drive to sign up volunteer observers. Two thousand people have been corralled into noting, among other things, when swifts arrive and ox-eye daisies flower. A torrent of existing records has also been released. This week, delegates at an EPN-organized conference in Wageningen are discussing how to standardize the measurements taken by the different European networks.

Phenology also helps to drive home messages about climate change. "Because the public understand these records, they accept them," says Sparks. It can also illustrate potentially unpleasant consequences, he adds, such as the finding that more rat infestations are reported to local councils in warmer years. And getting people involved is great for public relations. "People are thrilled to think that the data they've been collecting as a hobby can be used for something scientific — it empowers them," says Root.

Mary Manning is one such newly empowered observer. On Christmas Day she will go into her garden in Norwich, England, and record which species are flowering — just as she has every year since 1963. Manning, a retired schoolteacher, is surprised but delighted that researchers are interested in her records: "I did it because I loved doing it".

John Whitfield works in Nature's science writing team.

1. Sparks, T. H. & Carey, P. D. *J. Ecol.* **83**, 321–329 (1995).
2. Sagarin, R. & Micheli, F. *Science* **294**, 811 (2001).
3. Sparks, T., Roy, D. & Mason, C. *Essex Naturalist* No. 17, 31–37 (2000).
4. Sorenson, L. G., Goldberg, R., Root, T. L. & Anderson, M. G. *Climatic Change* **40**, 343–369 (1998).
5. Sagarin, R. *Nature* **414**, 600 (2001).
6. Worrall, J. *Nature* **399**, 101 (1999).

Nature's Calendar

♦ www.phenology.org.uk

European Phenology Network

♦ www.dow.wau.nl/msa/epn



Others caution that the mechanisms that link temperature and timing may not be straightforward. John Worrall, a forestry researcher at the University of British Columbia in Vancouver, points out that warmer weather may not necessarily delay autumn. Leaves age, and Worrall suggests that hotter summers could accelerate this

Patronage lies at the heart of Italy's academic problems

Funds are distributed through a system that runs on favours: nobody gets on without friends in high places.

Sir— Would the single key priority of transparent and modernized selection rules for directors of new Italian National Research Council (CNR) institutes identified in your Opinion article “A stain on Italian reforms” (*Nature* **414**, 133; 2001) provide a sufficient turning point for the current poor system? In Italy, there is a heavy tradition of centralized, ‘top down’ authority in the distribution of funding that has little to do with the quality of research.

Since 1995, owing to the apparent bankruptcy of our institute, we have been supported financially in our research only by organizations that have appreciated our work and reputation, and not by the CNR. The heart of the matter is a lack of healthy competitiveness for research funds in our country. A first step for change could be giving administrative and academic autonomy to individuals who are highly motivated and sufficiently skilled to do excellent research.

As you state in your article, funding at present is assigned through academic heads who sit on the commissions and share out the available posts and other resources on the basis of personal connections rather than on merit. In

this context, the challenge is to ensure a clear chain of responsibility from top to bottom and therefore to radically improve the research skills of the entire workforce of the CNR. This remedy is urgently needed.

Sad to say, academic promotion within the CNR is still based on recommendations and favours: a degrading spectacle including ‘vote exchange’ at all levels of promotion. This policy discourages innovation, as there is no clear career structure based on academic merit and results. If individuals with initiative and talent could at last enjoy their just rewards, the necessary profound renewal of the entire system might begin to happen.

As things stand, those without friends in high places remain barred from academic promotion, irrespective of their competence, and it can take four to five years to evaluate a candidate for promotion within the CNR.

Claudio Chiesa, Lucia Pacifico

Institute of Neurobiology and Molecular Medicine, National Research Council, c/o Institute of Pediatrics, “La Sapienza” University of Rome, Viale Regina Elena 324, 00161-Rome, Italy

CNR choices were made on the basis of capability

Sir— Your editorial “A stain on Italian reforms” (*Nature* **414**, 133; 2001) seems inspired by prejudice about some specific appointments made by the Italian National Research Council (CNR), rather than by the wish to understand the real basis of the decisions.

It is not true, as your article states, that the final decision on the appointment of institute directors is taken by the president. In fact, a board is in charge of each appointment; each member of the board votes for one of the candidates on a shortlist drawn up by a commission of peers on the basis of scientific achievement and managerial experience. The majority vote (where the president's vote counts for one) determines the choice.

In choosing new directors, the board has always taken into account the ability of the candidate to manage the transition from the old structure to the new one,

which requires skills in reduction and consolidation of the institutes as well as integration between different projects. Hence, our preferred candidates have excellent managerial experience and other personal characteristics, as well as distinguished scientific rank.

Finally, I fail to understand your statement that some choices derived from trading votes for favours among the members of the board. The great majority of the decisions were taken by consensus.

Lucio Bianco, President

CNR, Piazzale Aldo Moro, 7-00185 Rome, Italy

A challenge for research in Italy: to raise the dead

Sir— The dead body of Italian research was buried well before last month's mock funeral in Milan, reported in your News story “Funding fears spark Italian protest” (*Nature* **414**, 384; 2001). There is little to sob about in limiting the expansion of the

National Research Council (CNR) and of other public Italian research institutions that produce so little. One should, instead, complain about the Mafia-like rules afflicting Italian public institutions and determining the aberrant selection of candidates that will be such a negative influence on Italian science for years to come.

The recent failure to appoint high-profile scientists for several CNR institute directorships simply confirms what the vast majority of exiled Italian scientists have learned the hard way: nothing is really changing in the Italian science system. Therefore, it would have been wiser for Italian scientists working in public institutions to use their wit to change the rules. Public funding should be used to try to resuscitate the dead, not to refurbish the funeral parlour.

Arturo Sala

Molecular Haematology and Cancer Biology Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK

Open debate is essential on conservation issues

Sir— In their peevisish review of Björn Lomborg's book *The Skeptical Environmentalist* (*Nature* **414**, 149–50; 2001), Stuart Pimm and Jeff Harvey miss the main point just as they have in their comments in the same review on Julian Simon's 1996 book *The Ultimate Resource*. The main target of both books is the politicization of ecology that has created a dogmatic environmentalism.

In their rush to rubbish Lomborg's book, the reviewers perhaps missed the significance of Lomborg's title. What Lomborg, and Simon before him, describe is the continued disparity between apocalyptic claims for the future of mankind, with figures issued from large organizations such as the United Nations Food and Agriculture Organization, which often or even usually show the opposite.

Pimm and Harvey state that there are ecological laws that ensure the correctness of doom-laden predictions. Presumably one of these laws enabled the environmentalist Paul Ehrlich to state in his 1968 book *The Population Bomb*: “The battle to feed all of humanity is over. In the 1970s and 80s, hundreds of millions of people will starve to death.”

Laws do not exist in biology, only generalizations; there are exceptions to every biological principle. Extrapolating from the past to predict a doom-and-gloom future has been an industry from Malthus onwards. But the ultimate resource is the creativity and skill of the

human intellect; formulating the problem often generates solutions.

Democracy needs some people to shout loudly about the problems of the world in which we live, but such claims must be treated critically. That is Lomborg's thesis. Pimm and Harvey tell us that the main extinction threat is to species nothing is known about, which suggests these claims are hand-waving exercises. If nothing is known, how can extinction — or even teetering on the brink — be predicted?

If wilderness and species are to be saved from extinction, farming should be as efficient as possible. Excess agricultural land can then be returned to its original condition. Conservation is important, of course, but Pimm and Harvey's review suggests a common confusion with preservation. Human survival is the priority. Like all species in large numbers, our presence drives others to extinction. But new species will evolve to take advantage of the new environments created.

Open democratic debate about conservation policy is essential because there are many calls on public resources. The policies that are decided have to be the best return for money, and the public should vote on the outcome. In listing with glee the industry that will attempt to rubbish, instead of debate, Lomborg's book, Pimm and Harvey may have shot themselves in their feet. Such vehemence invites the conclusion that Lomborg (and Simon) have indeed exposed basic flaws in green political dogma.

Anthony Trewavas

Institute of Cell and Molecular Biology, Mayfield Road, University of Edinburgh, Edinburgh EH9 3JH, Scotland

Belief in our dominion is a backward step

Sir — It is disheartening to read your News item "Vatican approves use of animal transplants 'to benefit humans'" (*Nature* **413**, 445; 2001). It is the rationale of the Pontifical Academy, rather than its approval of xenotransplantation, that is particularly worrisome.

According to your report, "the academy argues that because humans enjoy a unique and superior dignity, and God has placed non-human creatures at the service of people, the sacrifice of animals is justified as long as there will be a 'relevant benefit for humans'"

This smacks of a return to pre-darwinian human arrogance and egotism. Didn't humanity long ago abdicate its monarchy over creation, giving up at last the notion of 'special creation' and human

'dominion over all things'? Even in the thirteenth century, St Francis of Assisi preached that all of nature, having been created by God, is important and worthy of respect.

I can think of few more dangerous attitudes than that promulgated in *Genesis* 1:28 and now by the Vatican, exhorting humanity, as the crown of creation, to "have dominion ... over every living thing". At what cost, mastery?

Robert C. Fleck

Physical Sciences Department, Embry-Riddle Aeronautical University, 600 S. Clyde Morris Blvd, Daytona Beach, Florida 32114, USA

It's Sulston all right — but not as we know him

Sir — Martin Kemp, in his "Science in Culture" article on Mark Quinn's *A Genomic Portrait*: Sir John Sulston points out that rather than a portrait, the artist has generated something akin to a relic (*Nature* **413**, 778; 2001).

In the artist's production of an artefact which may not be art, there is more than a hint of a parallel with Sulston's achievement. Here the scientist (and the Human Genome Project) has generated something which is not science, but a remarkable triumph of technology and organization — a catalogue of the human genome sequence. The interpretation of this triumph awaits the ingenuity of contemporary and future scientists.

Quinn has enlisted collaborators too; several million bacteria, some of which have been persuaded to take up fragments of Sulston's DNA. Like most representations, aspects of the subject will be missing from the finished work — unclonable portions of DNA which have not inserted into the bacteria. Artists consciously or unconsciously choose to emphasize certain features and downplay or omit others. In Quinn's case, he cannot direct the omission and lacks the means to decode the image for the observer.

Jerry Lanchbury

Molecular Immunogenetics Unit, Department of Rheumatology, Division of Medicine, GKT School of Medicine, King's College, London SE1 9RT, UK

Reality hits postdocs earlier in France

Sir — Your News story "Young, gifted ... and spurned" on the French postdoctoral system (*Nature* **414**, 145; 2001) portrays a one-sided view of the French system. Although your report makes some valid points, it fails to acknowledge that the UK

and US systems have many disadvantages, in the light of which it is not clear that the French system is worse.

In France, scientists gain tenure much earlier than their counterparts in Britain and the United States, which means that the former have more time and opportunity to formulate long-term research plans. Not every piece of research can generate several papers a year. Many topics, such as child development or ecology, need to be followed for years before a conclusion can be reached.

British and US postdocs generally waste a lot of potential research time writing proposals for renewal of short-term contracts. In the French system, postdocs are civil servants, so are less susceptible to having their research directed and can more easily conduct 'unfashionable' research without fear of losing next year's support.

The temporary contracts used in the UK and US systems mean that people can be forced to do 'trendy' research, or the research that one's professor or head of department wants done, to safeguard funding. This is not conducive to originality — it takes time to tell whether a piece of research is of fundamental importance. Bibliometric measures only tell us which topics are popular, but we could all be wrong, as history has often shown.

The deluge of postdoctoral workers in British and US universities stems from the often indiscriminate recruitment of PhD students essentially to do technicians' work but more cheaply and more disposably. Hence, after obtaining a PhD and holding a postdoctoral post or two, it becomes difficult to continue, because a disproportionately large number of qualified candidates are chasing a smaller number of vacancies. In France, it is difficult to find a job immediately after obtaining a PhD. So the difference between the two systems is that reality hits the researcher earlier in France.

There are more postdocs for each tenured post in the British system than in the French system. Even if there were not, it could be argued that the French system is fairer, as researchers know early on in their career if they can continue in scientific research, and are likely to be able to change careers more effectively than they can when they are older.

P.-L. Chau

Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, UK

correspondence

contributions to correspondence may be submitted to corres@nature.com. They should be no longer than 500 words, ideally shorter. Published contributions are edited for length.

Retrieving the 'eu' from eugenics

Can anything positive be rescued from the wreckage of the Nazi era?

The Unfit: A History of a Bad Idea

by Elof Axel Carlson

Cold Spring Harbor Laboratory Press: 2001.

451 pp. \$39

Eugenics: A Reassessment

by Richard Lynn

Praeger: 2001. 366 pp. \$85

Nick Martin

So what is to be done about eugenics? It is now almost universally reckoned to be a Bad Idea, as Elof Carlson's title makes plain. A book, a chapter, or even a seminar tut-tutting about all those famous supporters of eugenics who should have known better — from Beatrice Webb, H. G. Wells and Oliver Wendell Holmes to Julian Huxley, Peter Medawar and Francis Crick — is a sure step to success in today's politically correct academy. And those with the temerity to suggest that the large numbers of the Great and the Good who did support eugenics were not temporarily unhinged at the time should only do so from the safe haven of retirement (like Richard Lynn).

Nevertheless, the question of eugenics won't go away, as the arrival of yet two more books on the subject testifies. Both provide comprehensive histories of the eugenics movement from its founding in the 1870s by Francis Galton, motivated by lofty ideals for the improvement of mankind, to its horrific denouement in Nazi Germany. All this is well known, although Carlson unearths fascinating new material from the archives at Cold Spring Harbor Laboratory in New York, where the Eugenics Record Office was established in 1910. The purpose of the books, however, is to consider what, if anything, should be retrieved from the disastrous wreckage of the 1940s. Carlson thinks we risk our humanity by even contemplating the idea of 'unfit', whereas Lynn opines that authoritarian states that pursue eugenic policies (such as China) will end up dominating democracies that don't, so we'd better wise up.

The problem is with the 'eu', because if some people are well born, it implies that others are not, and nothing could be more offensive to the contemporary ear than such naked judgementalism. Scientifically there is a problem too, because evolution is value-free. The high-school dropout with six children by the age of 25 is clearly more fit (or 'better' in darwinian terms) than the career woman pregnant for the first time in her late thirties. If we would wish to alter the relative reproductive success of these two women to favour the latter, make no mistake that it is because we want it, not because Darwin, or

God, or the teleological destiny of man demands it.

Those who would shy away from the value judgements entailed in such a decision might reflect that this is the stuff of politics. For what is politics but the process of distributing finite resources among competing interest groups? And Lynn cites plenty of recent empirical data showing that reproductive rates are highly responsive to the granting or withholding of such resources as tax breaks for having children, maternity leave, or subsidized child care. So every politician is a eugenicist — it's just that they all have a different idea of 'eu' and 'non-eu'.

Of course, this is positive eugenics, its more benign side, in which those judged to have more desirable genotypes are given incentives to reproduce. Left at that, eugenics might never have been much more controversial than welfare, private schools or the UK National Health Service. The real problem is with negative eugenics, in which those deemed by someone in authority to have undesirable genotypes are discouraged or forcibly prevented from reproducing by compulsory sterilization. This was widely adopted in the 1920s in the United States and Europe. However, it was most enthusiastically put into practice by the Nazis at the same time that they were preparing plans for the mass-murder of homosexuals, Jews and Gypsies, and the same vile means were used to dispose of those they deemed genetically unfit. These terrible events, of which every generation needs to be reminded, have inextricably conflated the ideas of eugenics with genocide, and have forced suspension of any rational debate about the former for the past two generations.

It is the coercive aspects of eugenics that have so alienated a generation brought up on the assertion that individual rights take precedence over social rights. Nevertheless, the virtual elimination of public smoking in the Western world during the same period shows that large social changes can be achieved with clever publicity and modest coercion. In fact, some of the goals of negative eugenics are now being achieved in a voluntary way through the widespread provision of clinical genetics services in response to public demand. Any clinical geneticist will tell you that no one should underestimate the depth of parents' love for a disabled child. But equally, no one should underestimate their determination to avoid having another. Remarkably, the dramatic reduction in the US crime rate in the early 1990s has been linked to the legalization of abortion 18 years earlier. Is violent crime or



Bodily perfection? Miss Pulchritude, 1936 New York's most perfect model, poses with Mr Muscles.

abortion on demand the worse social evil?

Given the importance of the topic, there should be good data on the reproductive patterns of contemporary human populations, but there are not. Most of the claims made by Lynn for dysgenic effects rely on differential family size with respect to educational or income levels. Yet reproductive fitness must be measured from zygote to zygote, and few have had the patience or resources to collect data on reproduction in the second generation. A study from the 1930s suggested that children from large families were less likely themselves to reproduce, all but cancelling out the effect of large family size, but this has never been properly replicated.

We know virtually nothing about the nature of fertility differentials in Western societies except that they exist to a striking degree — up to 28% of Australian women, for example, are now childless at menopause. Variation in reproductive fitness is surprisingly heritable — up to 40% in recent Australian and Danish studies. In 1929, R. A. Fisher showed that the additive genetic variance in reproductive fitness was a direct measure of the rate of evolution. This suggests that our populations are currently experiencing quite drastic natural selection. But for which traits? We have little idea, but the old eugenicists might not like the answer. ■

Nick Martin is at the Queensland Institute of Medical Research, Brisbane 4029, Australia.

BETTMANN/CORBIS

The three ages of fire

Fire: A Brief History

by Stephen J. Pyne

British Museum Press: 2001. 204 pp.

£15.99

C. K. Brain

How did humanity attain its dominance in the natural world when fossil evidence indicates that, a million years ago, our ancestors were subservient to the great cats of Africa? The answer must surely lie in the blossoming of human intellect and its highly effective expression through culture and technology.

Of all the important items in the hominid cultural tool-kit, none has been more crucial than the management of fire; in fact, the first step taken by our ancient ancestors to overcome the ever-present threat posed by powerful predators was very likely the management of fire at their camp sites. This does not imply that those pioneering and imaginative entrepreneurs knew how to start a fire at will — presumably they gathered burning branches from lightning-induced fires and took them back to their sleeping places. Imagine the frustration that must so often have been

experienced when, on waking in the morning, people found that their fire of the night before had died and there was no obvious way to resurrect it!

It is hardly surprising that fire came to be regarded as a magical substance, to be treated with the greatest care and reverence. Since those early days, many people have been obsessed by fire and by the never-ending ramifications into which the technology of fire has led them.

One such is Stephen Pyne, an environmental historian who has spent much of his productive career researching the topic of fire and writing extensively about it. The synthesis — *Fire: A Brief History* — is his latest contribution to a six-volume series entitled "Cycle of Fire", in the Weyerhaeuser Environmental Books series, published by the University of Washington Press under the general editorship of William Cronon, who also contributed a foreword to each book. The previous volumes have explored a wide variety of themes, but the essence of these has been distilled in this brief history in a flowing and delightfully readable form.

Although fire appeared on Earth long before the coming of humans, it has not always been a feature of this planet. Combustion, in the sense referred to here, requires the presence of oxygen, and this was not an important component of the early atmosphere. It slowly

accumulated as a photosynthetic by-product of cyanobacteria, algae and, later, plants. Initially, an abundance of reduced iron and other material in Precambrian waters would have absorbed any oxygen released, so it was only about 2.3 billion years ago that free oxygen formed a significant component of the air. Another obvious requirement for fire is the presence of combustible fuel and this, too, was the product of living organisms. So it is true to say that fire was born of life and has been its companion for at least 400 million years, when charcoal makes its appearance in the fossil record during Devonian times. Such burning would have resulted from what Pyne refers to as "first fire" — combustion caused by lightning or the heat of volcanic action. "Second" and "third" fire had to await the appearance of the hominids.

For hunter-gatherers, managed fire would have cooked their food and dislodged unwanted predators from dense vegetation around their camp sites. But it was when people took to agriculture that fire became a necessity. It literally cleared the way for the planting and harvesting of crops. This was "second fire" and, as it spread around the world, it told of the presence of agriculturalists, who changed the nature of the world's habitats for ever.

With agriculture came an unprecedented growth of human populations and the establishment of cities. Here, in an industrial context, "third fire" was destined to flourish. It relied very largely on the combustion of fossil fuels — coal, oil and gas — in which the energy of age-old sunlight had been stored by once-living organisms. This energy could be released again through combustion but, in the industrial context, flames would seldom be seen. Release would generally occur in confined spaces, such as within an internal combustion engine, or at a power station where electricity is generated for a multitude of human needs. People have come a long way from the time of their first fires; now, for instance, it is said that 'superior fire-power' is likely to be the deciding factor in any military encounter.

This historical account of fire, ancient and modern, presented so well by Stephen Pyne, allows the reader to view an essential component of civilization with new insight and enlightenment. ■

C. K. Brain is Emeritus Curator at the Transvaal Museum, PO Box 413, Pretoria 0001, South Africa.



To the ends of the Earth

Inhabitants of the Yap Islands in the Pacific Ocean fish the inshore coral reef. This is one of many photographs charting 15 years of voyages by the ship *Explorer* to be found in *Ship in the*

***Wilderness: Voyages of the MS "Explorer" Through the Last Wild Places on Earth* (Gaia Books, £19.99, \$35). Photographs are by Jim Snyder, with narrative by Keith Shackleton.**

More on fire

Flammable Australia: The Fire Regimes and Biodiversity of a Continent

edited by Ross A. Bradstock, Jann E. Williams & A. Malcolm Gill

Cambridge University Press, £90, \$130



PAOLO COCCO, POOL/AP

Nodding heads: the Pope and President Bush discuss the use of human embryos in research.

Navigating a delicate dilemma

The Human Embryo Research Debates: Bioethics in the Vortex of Controversy

by Ronald M. Green

Oxford University Press: 2001. 231 pp.
£22.50, \$29.95

Justine Burley and Alan Colman

A more apt subtitle for this excellent book might have been 'Bioethics into the Black Hole'. Ronald Green, a considered thinker, bears credible witness to the lamentable standard of argument, the subterfuge of lobby groups and the ruthless politicking that has dominated Washington-based policy-making over embryo research and related scientific developments.

In the book's first chapters, Green charts the deliberations of the Human Embryo Research Panel, established by the National Institutes of Health (NIH) in 1993. Here he interweaves recent history with sound philosophical reflection, painstakingly laying out the arguments — scientific, legal and ethical — behind the group's final recommendations, which supported the funding of research on embryos for specific purposes. This serves to air the contents of the panel's

1994 report, which was neutered by presidential decree and swiftly forgotten — evidently a source of dismay for Green. It also enables the author to introduce some of his own views on human embryo research. The most important of these is that any assessment of the moral status that should be accorded to early human life is of necessity based on existing moral doctrines. Revolutionary this is not; nonetheless, it is striking to see how little appreciated its practical implications are.

Green argues that publicly justifiable policies on embryo research cannot be arrived at simply by invoking a particular set of objective properties for the embryo. It is, for example, asserted that a human life begins at conception, and that from this fact it follows that the protection afforded adult humans should also be enjoyed by the fertilized egg. Likewise, at the other end of the debate spectrum, it is held that no human individual should be accorded basic rights until that individual has become a person — usually defined as someone with the rational capacities for self-awareness and self-direction. But there is no definitive reason for accepting either of these positions. Hence policy cannot be made through bald appeal to them.

In a pluralistic society it is impossible to reconcile divergent metaphysical conceptions of when life has value. Instead, policy must be derived by reference to the prevailing political culture as well as to people's shared principles of practical reasoning. In the case of embryo research, the first step towards an acceptable policy is to identify the diversity of ideas held by society about an embryo's status. We should then reflect on these in relation to shared values in conceptually related areas, the purposes of the embryo research, whom it could benefit, how much the suffering of people who might be helped by the research matters to us, and so on. What should then emerge is a policy that can be accepted in a pluralistic society by reasonable individuals. The requirement of reasonableness means that we understand that on subjects where people have divergent, deep-seated moral convictions, a policy may never fully reflect all aspects of our respective moral doctrines, but will fit sufficiently with our commonly held values to stand as legitimate public policy.

Green makes three chief observations about how policies on embryo research are typically made: that recommendations are not adequately backed up by reasoned justifications; that panel members and witnesses are frequently lobbyists and/or lack expertise; and that committees tend to endorse the decisions they perceive to be politically expected of them. The Human Embryo Research Panel was not seriously afflicted by any of these shortcomings, and helpful references to its proceedings resurface in subsequent chapters of the book.

When Green tackles the issue of human

reproductive cloning, he does not criticize the 1997 conclusions of the National Bioethics Advisory Commission (NBAC), which was entrusted with investigating it, but the way they were reached. He charges the commission with being an obedient child of the Clinton administration, with over-representing religious lobby groups, and with sloppy thinking. For example, in response to the argument that a cloned child, however abnormal, is better off existing than not, the NBAC report says that metaphysical considerations of non-existence versus existence lead to absurd conclusions and so should be avoided. Yet the report goes on to recommend that the present moratorium on federal funding for cloning should continue — and requests voluntary compliance from the private sector — on the grounds that a child is better off not existing than being harmed by the technique that could bring it into existence.

Green notes that the commission's preoccupation with physical safety is contradictory. In effect, the health of any future clone is placed at risk by denying federal funding for the embryo research needed to conduct a rigorous appraisal of the technology. Otherwise, assessment of the technical problems of cloning is left to the private sector, which is largely exempt from regulation.

While the NBAC was deliberating over stem-cell research in 1998, so too was Green, as a member of a working group of the American Association for the Advancement of Science (AAAS). Federal funding for embryo research had been outlawed in 1996. But when James Thomson and John Gearhart published their respective successes in cultivating immortal human embryonic stem-cell lines in 1998, the push to change the law became concerted and highly politicized. In 1999 the NBAC recommended that, because there was a public consensus, the law should be relaxed to allow public funding for embryonic stem-cell work. But Green states that there was no such public consensus. He also says he suspects that the NBAC's commissioners travelled this ill-fated route because they were uncomfortable with the tactic deployed by the NIH and the AAAS of recommending that embryonic stem cells should be used but not derived in the public sector.

Green accepts the moral validity of the use-versus-derivation distinction. He argues that society's needs can be met by the use of spare embryos from *in vitro* fertilization procedures, and that we have a duty to minimize, within reason, any upset to people's moral sensibilities. This, we are told, translates into a policy that supports federal funding for use but not derivation of stem cells.

Green's stance here is problematic — for one thing he fails to give a cogent rebuttal of the 'dirty hands' objection — and it reflects a wider difficulty with his thinking about policy-making on embryo research. Events roughly coinciding with the publication of

his book indicate that piecemeal policy-making may be misguided in areas of science where developments are rapid, and vulnerable to the vagaries of politics. The use-versus-derivation approach vaunted by the NIH and AAAS was hoist by its own petard. Already schizophrenic, in the hands of President Bush it became even more bizarre when he decreed that only existing embryonic stem-cell lines could be researched with public monies. Not all of these cell lines have been published, nor have they been adequately characterized. Moreover, they were created using mouse feeder cells which, according to guidelines of the Food and Drug Administration, would render cells derived from them xenografts, not purely human.

Because of the huge potential benefits of research on human embryonic stem cells and because so little is known about them, a sensible policy should be wide, not narrow, in its scope. Of course, the NIH and AAAS (and arguably Bush) strained every nerve to ensure that public money for embryonic stem-cell research was made available. But Green's criteria for sound policy-making were not satisfactorily met in the process. Green, perhaps, 'went native' in Washington, despite himself.

Serious readers of science, philosophy, law and government who are interested in medical research cannot fail to enjoy *The Human Embryo Research Debates*. It should be required reading for students across these disciplines, not to mention for those on the right of so-called 'abortion politics' — if they care about the arguments, that is. ■

Justine Burley is at Exeter College, University of Oxford, Oxford OX1 3DP, UK. Alan Colman is at PPL Therapeutics, Roslin Institute, Edinburgh EH25 9PP, UK.

More on the embryo debate

The Human Embryonic Stem Cell Debate: Science, Ethics, and Public Policy

edited by Suzanne Holland, Karen Lebacqz & Laurie Zoloth

Cambridge University Press, £90, \$130

What if Mendel had studied sheep?

Genetic Prehistory in Selective Breeding: A Prelude to Mendel

by Roger J. Wood & Vítězslav Orel
Oxford University Press: 2001. 342 pp.
£49.50, \$85

William G. Hill

Successful improvement of crops and livestock was carried out long before Mendel elucidated the principles of inheritance, and the effectiveness of artificial selection was a major contributor to Darwin's theory of evolution by natural selection. Quite simply,

as offspring resemble their parents, selective breeding usually provides results. Indeed, simple selection of the best performers is still the main component of most animal breeding programmes for major traits such as growth rate. But we can now use Mendel's laws to explain why it works and is an efficient practice.

In *Genetic Prehistory in Selective Breeding*, Roger Wood, of the University of Manchester, and Vítězslav Orel, emeritus head of the Mendelianum in Brno, aim to set the scene for Mendel's work, particularly in the context of ideas and practice in animal breeding in central Europe. The superior fine wool of the Spanish merino sheep was recognized in the textile industry; but, in an old version of the nature–nurture debate, an important question was the extent to which its quality would be retained in different environments and in successive crosses to local breeds. Among those interested was C. F. Napp, who was abbot of the monastery at Brno when Mendel entered it in 1843 and throughout the time of the latter's famous work. Napp participated in the discussions of the local sheep-breeders' society, was interested in the scientific basis of improvement and was presumably a stimulus to Mendel.

In the later part of the eighteenth century, Robert Bakewell in England had demonstrated the effectiveness of selection — aided by inbreeding — to fix qualities, with great success and to widespread acclaim. His influence was substantial and many interested breeders, including some from central Europe, visited him. Bakewell, however, had

improved meat production in sheep and so it was not certain that his methods were relevant to improving wool quality and yield. Wood and Orel's chapters on Bakewell's ideas and work are, in themselves, a fascinating story, although better known than that of the work of breeders in mainland Europe.

While the problems of sheep, and specifically wool, improvement were presumably a stimulus to Mendel, both his and his abbot's interests were wider. Napp, for example, was also president of the area's Pomological and Oenological Society. Perhaps more significantly for the advancement of science, Mendel conducted his crossing experiments on traits with discrete classes — the tall and dwarf peas had a non-overlapping distribution. He would almost certainly have got nowhere studying a trait such as wool diameter, which has a continuous distribution and no clearly segregating classes in a cross.

Although I do not find Wood and Orel's implicit thesis of the important influence of sheep-breeding on Mendel's work wholly convincing, it is nevertheless both an interesting and a stimulating study. They have undertaken an impressive amount of research in the archives on sheep-breeding in Europe in the eighteenth and nineteenth centuries, and presented their findings and conclusions clearly and logically. I learnt a lot from the book. ■

William G. Hill is at the Institute of Cell, Animal and Population Biology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, UK.



A trip to the sea

Dawn, and a clutch of hatchling green sea turtles (*Chelonia mydas*) emerge from their nest on a beach on Sipadan Island, Borneo, to make for the relative safety of the sea. *Turtles, Tortoises & Terrapins: Survivors in Armor*

by Ronald Orenstein (Firefly Books, \$45) gives a comprehensive coverage of the many other species of these shelled animals — their evolution, life history and conservation.

Weaving a social web

The Internet promises to revolutionize public engagement with science and technology.

David Dickson

Modern communications technologies — in particular the Internet — have revolutionized the communication of scientific knowledge. But most interest has so far focused on their impact on the exchange of information between professional researchers, such as the development of electronic publication and the use of massive online databases.

Relatively little attention has been paid to the equally important issue of how these same communications technologies could transform the way in which this knowledge is disseminated to non-scientists, and the possibilities that open up for a new approach to the public understanding of science.

This approach offers an alternative to both the 'top-down' preaching and the carefully orchestrated 'dialogues' between scientists and 'the public' that are currently the most favoured forms of communication. In contrast, it promises a more grass-roots engagement in debates on the promises and limitations of modern science and technology.

The current imbalance in the use of the Internet to communicate scientific knowledge is understandable. The ease with which data can be shared electronically between researchers has many immediate and positive consequences for those both receiving and transmitting the information. This has encouraged significant investment.

By contrast, the use of the Internet to promote a true public understanding and appreciation of science faces significant hurdles. One is the question of access. It is ironic that those who stand to benefit most from the information that the Internet can provide are often those who lack the technical or financial resources to access it.

A second hurdle is that many efforts to use the Internet to communicate information about science and its impact on society have an implicit agenda. This is as true for the pressure groups that have so successfully used the web to promote campaigns on particular issues — from genetically modified

crops to climate change — as it is for corporations that seek to counter what they characterize as 'propaganda' by mounting their own websites to present 'the facts'.

These hidden agendas can create their own problems. Think, for example, of South Africa's president, Thabo Mbeki, who claimed to have come across a significant scientific controversy over the relationship between HIV and AIDS during a late-night Internet surfing session when he happened upon a website that challenged the prevailing scientific consensus.

And then think not only of the way that some South African officials have used their president's views to justify limiting their efforts to combat HIV, but also of how they have accused manufacturers of anti-HIV drugs of using the web to try to stifle scientific debate in order to promote products in which they have a commercial interest.

We all stand to benefit from the exploration of ways to use the power of the Internet to disseminate reliable scientific and technical information. The effectiveness of a group engaged on any side of a public debate can only be enhanced by a sounder awareness of the scientific and technical dimensions of the disputed topic.

At the same time, as the case of HIV/AIDS underlines only too dramatically, we also need ways to ensure that the potential of the Internet for enhancing access to scientific information is exploited responsibly. Not because doing otherwise is morally damaging — there is no room here for censorship — but because, in the long run, it is counter-productive.

Electronic scientific publication is now being recognized as requiring rules and procedures to ensure its legitimacy, even if these are different from those in place for printed scientific publications (see www.nature.com/nature/debates/e-access). The same is true for electronic dissemination of information about science and technology, particularly when the information is of direct relevance to contentious issues.

There is a particular opportunity here to use the Internet to meet the social and economic needs of developing countries. These countries stand to benefit more than anyone from the reduced marginal costs of electronic dissemination — but also face many difficulties created by the continuing existence of a 'digital divide' between rich and poor.

Many experiments are needed to find the best way of doing this. One is *SciDev.Net*, a website, launched this week, devoted to delivering news and providing a forum for debate about the contribution — real, potential or



Outreach: how can scientific information best get to those who need it most?

contested — of science and technology to the needs of developing countries.

The website is being launched with the editorial backing of the journals *Nature* and *Science*, both of which have agreed to provide free access each week to relevant articles (selected by *SciDev.Net* editorial staff). It is being produced with the support of the Third World Academy of Sciences, and is financed by international aid agencies in Britain, Sweden and Canada.

Much of the new website uses a traditional approach to providing information about science, by publishing news items, features, contributed opinion articles and 'policy briefs', albeit in electronic form. In some cases, this material will be linked together in 'dossiers' aimed, for example, at policy-makers and others looking for an authoritative overview of a topic.

The site will also seek to exploit new opportunities opened up by the web. It will, for example, allow instant comment and discussion about any item published on the site, and also provide a guided tour, using annotated links, of other relevant websites.

A key challenge to initiatives such as *SciDev.Net* is how to democratize the gathering and dissemination of information about science in a way that retains the legitimizing devices of conventional editorial processes.

Providing this can be done, responsible and imaginative use of the Internet is an exciting way of matching the resources offered by science and technology directly to particular social needs. It is a possibility from which we all stand to benefit — but particularly those now excluded from enjoying the fruits of modern science in developed and developing countries alike.

David Dickson is the founding director of *SciDev.Net* and a former news editor of *Nature*.

▶ www.scidev.net

Developing countries stand to benefit most from electronic distribution of information.

Light work with water

Nathan S. Lewis

Sunlight can be harnessed by semiconductors to generate a fuel, hydrogen gas, from water. This approach will be impracticable until certain materials-related constraints are overcome: photochemists are on the case.

A stable and efficient material that uses sunlight to split water into hydrogen and oxygen would be an immense blessing. Water and sunlight are both renewable resources, and cheap. And one of the end products, H_2 , is a clean-burning fuel that produces water as the waste product. Researchers are on the case in searching for such a material¹ and — writing on page 625 of this issue² — Zou *et al.* describe a step along one way towards this Holy Grail of inorganic photochemistry.

There are three fundamental requirements for any system for converting and storing solar energy³. First, sunlight must be efficiently absorbed to produce excited electron states in the light-absorbing material, the photocatalyst. Second, to obtain directed work, either chemical or electrical in form, the photoexcited electron and its accompanying electron vacancy must be separated in space to prevent their recombination, which produces heat and wastes energy. Third, the photoexcited charge must be energetically and kinetically able to perform a desired chemical transformation, for instance splitting water. Furthermore, these charges must not result in undesirable end products, such as heat, or chemically transform or otherwise degrade the photocatalyst. Satisfying all of these requirements simultaneously is a tall order.

A popular approach is to use semiconductors as the light absorbers. Semiconducting solids generally have broad, strong optical absorption characteristics, meeting the first requirement for solar-energy conversion. They also generally satisfy the second requirement, because effective charge separation is facilitated by electric fields at the interface between a semiconductor and selected liquid electrolytes. The electrolyte provides the chemical feedstock that will be transformed into the fuels formed by the photochemical reaction, and ensures that electrical charge can flow through the liquid to complete the electrical circuit of the water-splitting electrolysis cell. Figure 1 shows various strategies for converting energy from the Sun, with the semiconductor–electrolyte cell in the centre.

The key requirement is the third one: whether the photoexcited charge can split water efficiently. Back in 1972 a seminal paper⁴ described the sunlight-assisted electrolysis of water, using crystalline TiO_2

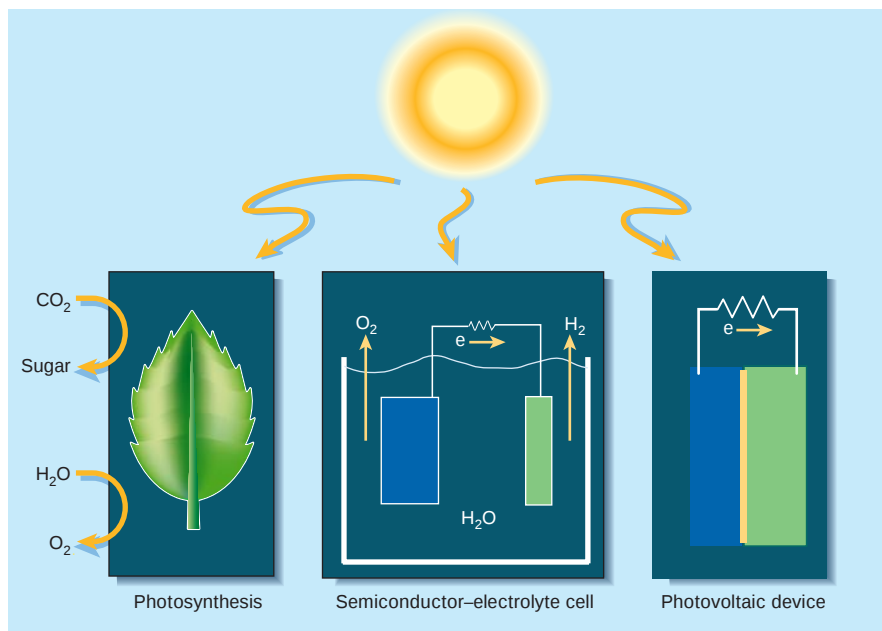


Figure 1 Energy-conversion strategies for creating fuel or electricity from sunlight. Left, in photosynthesis, plants use solar radiation, in conjunction with CO_2 and water, to produce sugars (the fuel) and O_2 . Right, photovoltaic devices can convert solar energy directly into electricity: when light shines on a photovoltaic solar cell, electrons are released from a semiconducting material (blue), and then flow as electric current to a metal electrode (green). Centre, the system used by Zou *et al.*², which produces H_2 as the potential fuel. The semiconducting material and metal electrode are immersed in water. Under light irradiation, photoexcited electrons reduce water to give H_2 , whereas the electron vacancies oxidize water to O_2 . Zou *et al.* have doped an indium–tantalum–oxide with nickel, and find that this material absorbs light in the visible spectrum, an advance over previous photocatalysts.

photoelectrodes, and prompted a flurry of research into photoelectrochemical water splitting. In consequence, several metal oxides, including $SrTiO_3$ and $KTaO_3$, were identified^{3,5} as being able to sustain the light-driven, unassisted photoelectrolysis of water into H_2 and O_2 .

Unfortunately, a fundamental problem has thwarted the practical implementation of such water-splitting systems. Although $SrTiO_3$ and $KTaO_3$ effectively convert absorbed photons into stored chemical fuel, the band gaps of these metal oxides — that is, the energies at which light starts to be absorbed — are too large to allow the efficient absorption of most of the photons in the solar spectrum. The overall energy conversion efficiency of such systems is only 1–2%. Compounds such as $CdTe$ (a so-called metal chalcogenide) or InP (a type III–V semiconductor) have smaller band gaps that are better matched to the spectral distribution of sun-

light reaching the Earth, but these materials either corrode or become inert when used as photoelectrodes in aqueous solution^{3,5}. When the band gap of various metal oxides is made smaller (to obtain more overlap with the solar spectrum), as it is in ZnO or Fe_2O_3 , the potential of the photoexcited electron in the semiconductor becomes more positive than the potential needed to reduce water to H_2 , and the reaction becomes thermodynamically unfavourable at a 'room' pressure of one atmosphere⁶.

In short, the problem is this: the materials that are stable in water and can split water into H_2 and O_2 do not absorb sunlight effectively, and the materials that absorb sunlight effectively cannot sustain photochemically induced water-splitting. This 'duality principle' limitation of photoelectrolysis does not reflect a fundamental thermodynamic barrier, because a material with a band gap of 1.5–1.6 eV is capable of providing the 1.23 V

of free energy needed to electrolyse water at one atmosphere of pressure. Instead, the constraint is imposed by the interplay between the optical, electronic and chemical properties of the light-absorbing materials that have been tested.

The work of Zou *et al.*² offers hope that these materials-related constraints can be overcome. The authors report that introduction of Ni into InTaO₄ extends the light absorption of the composite In_{1-x}Ni_xTiO₄ photoelectrode family into the visible region of the solar spectrum, and that the photo-excited electrons still retain enough energy to reduce water to H₂. The photogenerated electron vacancies then oxidize water to O₂, completing the circuit and driving the water-splitting reaction. Zou *et al.* show that in this system wavelengths as long as 420 nm can power a water-splitting process. They consider the system to be stable, because the amount (in moles) of H₂ and O₂ produced exceeds the amount of reducing equivalents in the photoelectrode sample.

This, however, is far from the end of the story, because the conversion efficiency of Zou and colleagues' system is less than 1%. The next steps involve extending the approach to produce materials that absorb still more of the visible region of the spectrum. The optimum band gap for a single-threshold device to convert sunlight into stored energy is between 1.1 and 1.7 eV, with the higher end of the range required for water splitting because of the energetic constraints that accompany the formation of H₂ and O₂ at a pressure of one atmosphere³. The limits to which the existing family of metal oxides can be pushed while simultaneously satisfy-

ing the energetic and light-absorption constraints are not well understood theoretically or experimentally. So advances in this area will be essential for developing an integrated photocatalytic system for converting and storing solar energy.

It is encouraging that all of the parts of a water-splitting process exist in biological systems: photosystem II in plants produces O₂ from water; hydrogenase enzymes reduce water to H₂; and the chlorophyll-derived energy-harvesting and charge-separating components of plant and bacterial photosynthesis provide a model for efficient light absorption and charge separation structures that can drive fuel-forming photochemical reactions. Plants, however, are far from being optimal machines for converting solar energy: only 3–4% of the total sunlight energy falling onto a leaf is converted into stored free energy by photosynthesis. Artificial photosynthetic systems are inspired by the biological process, but their goal is to outperform it. So although the results of Zou *et al.*² are promising, there's a way to go before we can beat nature. ■

Nathan S. Lewis is in the Division of Chemistry and Chemical Engineering, California Institute of Technology, 210 Noyes Laboratory, 1200 East California Boulevard, Pasadena, California 91125, USA.

e-mail: nslewis@its.caltech.edu

1. Bard, A. J. & Fox, M. A. *Acc. Chem. Res.* **28**, 141–145 (1995).
2. Zou, Z., Ye, J., Sayama, K. & Arakawa, H. *Nature* **414**, 625–627 (2001).
3. Tan, M. S. *et al. Prog. Inorg. Chem.* **41**, 21–144 (1994).
4. Honda, K. & Fujishima, A. *Nature* **238**, 37–38 (1972).
5. Finklea, H. A. *Semiconductor Electrodes* (Elsevier, Amsterdam, 1998).
6. Kung, H. H., Jarrett, H. S., Sleight, A. W. & Ferretti, A. J. *Appl. Phys.* **48**, 2463–2469 (1977).

of the immune system, thus triggering various immune responses. The genes that make up the MHC are highly 'polymorphic', with different individuals carrying different combinations of these variable genes. These genetic variations alter the encoded proteins so that they differ in a few amino acids around the peptide-binding site, and can bind different foreign peptides.

The MHC genes form a reliable marker of individuality at the cellular level — for example, the degree of similarity between the MHC genes of different humans is what determines whether or not a tissue transplant will be rejected. Moreover, mice can be trained to distinguish between the odours of urine from individuals that are genetically identical except for their MHC³. Yet the mechanism by which MHC type influences urine odour is still unclear, and other genetic differences also influence the ability of mice to discriminate between urine odours^{4,5}.

By far the most abundant proteins in mouse urine are the major urinary proteins (MUPs). They are excreted in milligram quantities per millilitre of urine — a concentration at least 100,000-fold higher than that of MHC protein fragments. They are members of the lipocalin family of proteins, which bind small volatile molecules in urine⁶ and release them over prolonged periods from dried urine marks⁷. But MUP proteins are far more diverse than they would need to be if they were simply a reservoir for urinary volatiles. Like the MHC genes, MUP genes are polymorphic, and urine from a wild mouse contains a mixture of about 7–12 different MUPs. Hurst *et al.*¹ now show that the urinary MUP profile can convey information about the identity of the producer in the context of the territorial behaviour of male mice.

Mice have complex social structures in which a dominant male will defend a territory from intruding males, and mark it by depositing small quantities of urine on solid surfaces. A resident male will also counter-mark any urine marks left by intruding males, advertising both his presence and his dominance⁸. It has been shown⁹ that the airborne volatiles attract males to investigate the marks, whereas the counter-marking is driven by the protein component of the marks. Counter-marking relies on the male's ability to distinguish his own marks from those of competitors. Hurst *et al.*¹ looked at the involvement of MUPs in this behaviour.

The authors first analysed the behaviour of males in response to urine marks left in their territory by different intruding males. They found that when resident males were presented with urine marks from a brother with a different MUP profile, the animals increased both the length of time that they investigated the marks and their rate of counter-marking — despite the fact that the

Animal behaviour

How mice make their mark

Peter Brennan

The social behaviour of many animals relies on their ability to use odour cues to distinguish among individuals. Studies of mice highlight the importance of urinary proteins in this complex signalling system.

For many animals, chemicals present in skin secretions, sexual secretions, saliva or urine are important signals, conveying information such as gender, sexual maturity, health, social dominance and individual identity. Some of these chemical cues are derived from interactions with the environment — in the shape of gut bacteria or diet, for example — whereas others are encoded by genes. So far, much of the research in this area has been directed towards the signals of individuality that are involved in sexual behaviour, enabling animals to choose an appropriate mate and avoid inbreeding. But signals of individuality are also important in social behaviour, such as nepotism

and territoriality, although their chemical basis is unclear. Hurst and colleagues¹ tackle these issues on page 631 of this issue, and provide evidence that major urinary proteins determine the individuality of the urine marks used in the territorial behaviour of mice.

The search for chemical identity signals has generally been focused on genes of the major histocompatibility complex (MHC), because there is substantial evidence that mice prefer to mate with partners of dissimilar MHC type². The MHC is a cluster of genes that code for cell-surface proteins. The function of these proteins is to bind peptides from invading pathogens and present them to cells

intruding male was familiar to them. In contrast, urine marks from a brother with the same MUP profile did not elicit any increase in investigation or counter-marking. These results suggest that the key determinant of urine-mark identity is the MUP profile, rather than the MHC or other genes, which would be expected to differ among brothers.

The importance of MUPs is supported by Hurst *et al.*'s second experiment, in which they changed the MUP profile of the resident male's own urine by adding an artificially produced MUP protein. The adulterated urine did not elicit more investigation, suggesting that the MUP-bound volatiles in the urine were unchanged. However, the males did increase their rate of counter-marking, consistent with previous findings⁹ that it is the protein — rather than the volatile — component of urine that drives counter-marking behaviour (although the mechanisms are not known).

So where does this leave the MHC genes in determining the individuality of odours? It remains to be seen whether the ability of mice to discriminate between urine odours on the basis of MHC type³ has any behavioural significance in the wild. Even if it does not, that does not necessarily negate the importance of the MHC — odour cues from different sources might be involved in different behavioural contexts. For example, mate choice could turn out to be much more dependent on the individuality of sexual or skin secretions than on urine, and these odour cues might be related to MHC type.

Finally, although Hurst *et al.*'s findings¹ relate specifically to the counter-marking behaviour of male mice, MUP profiling and the use of artificially produced MUPs provide the tools with which to investigate the involvement of these proteins in other aspects of mouse behaviour. The results should also be of interest to the growing band of researchers who are trying to link genes and behaviour by studying genetically manipulated mice. For such a widely used laboratory animal, surprisingly little is known about the invisible chemical cues that guide its natural behaviour, and these cues are often overlooked when interpreting the subtle effects of genetic manipulation. ■

Peter Brennan is in the Sub-Department of Animal Behaviour, University of Cambridge, High Street, Madingley, Cambridge CB3 8AA, UK.

e-mail: pab23@cus.cam.ac.uk

- Hurst, J. L. *et al.* *Nature* **414**, 631–634 (2001).
- Penn, D. J. & Potts, W. K. *Am. Nat.* **153**, 145–164 (1999).
- Yamaguchi, M. *et al.* *Proc. Natl Acad. Sci. USA* **78**, 5817–5820 (1981).
- Singh, P. B. *Reproduction* **121**, 529–539 (2001).
- Singer, A. G., Beauchamp, G. K. & Yamazaki, K. *Proc. Natl Acad. Sci. USA* **94**, 2210–2214 (1997).
- Böcskei, Z. *et al.* *Nature* **360**, 186–188 (1992).
- Hurst, J. L., Robertson, D. H. L., Tolladay, U. & Beynon, R. J. *Anim. Behav.* **55**, 1289–1297 (1998).
- Hurst, J. L. *Anim. Behav.* **40**, 209–222 (1990).
- Humphries, R. E., Robertson, D. H. L., Beynon, R. J. & Hurst, J. L. *Anim. Behav.* **58**, 1177–1190 (1999).

Astronomy

Gravitational microlens in motion

Andrew P. Gould

Astronomers have now imaged a stellar microlens as it speeds across the sky. A similar effect will soon allow them to measure the mass of a lens, whether dark or luminous.

In 1919, Arthur Eddington led a famous expedition¹ to measure how much the Sun deflects light from distant stars during an eclipse. His results confirmed Einstein's prediction that gravity could bend light. This effect causes 'gravitational lensing', in which starlight is deflected by a suitable 'lens' — the Sun in Eddington's case. The lens can also be a distant galaxy or a nearby stellar-mass object. If the distorted images are so close together that they cannot be resolved as separate images, the effect is known as microlensing.

Fifty years elapsed before Sjur Refsdal² suggested the first practical application of gravitational lensing: measuring the masses of nearby stars. On page 617 of this issue Alcock *et al.*³ bring us a step closer to fulfilling Refsdal's dream by taking the first direct images of a gravitational microlens, which they discovered eight years ago. In doing so they have unified astrometry — precision measurements of stellar positions — and gravitational lensing, opening up a whole new area of astronomy.

Refsdal argued that applying a simple series of astrometric measurements to a gravitational lens provides a means of determining the mass of the lens. The deflection of starlight from a distant star (the source) by a nearby star (the lens) is proportional to the lens mass and is related to two astrometric quantities: the relative positions of the lens and source, and their relative parallax. Parallax is the apparent motion of a star on the sky caused by the motion of the Earth around the Sun, and is inversely proportional to the star's distance. Parallax is therefore the standard method of determining distance to a star, and relative parallax is simply the difference in parallaxes of two neighbouring stars. So, to find the lens mass, astronomers need to know the relative parallax of the lens and source, their relative positions, and the strength of the lens (how much it deflects starlight).

In 1993, when the MACHO⁴ and EROS⁵ collaborations announced the first detections of gravitational microlensing, the goal was very different: they were searching for the mysterious 'dark matter' that is thought to account for 90% of the mass of galaxies and as much as 99% of all the matter in the Universe. A few years earlier, Paczynski⁶ had suggested that if dark matter in our Galaxy were composed of brown dwarfs, black holes or any other massive compact objects (later

dubbed MACHOs) with masses in the range of 10^{-6} to 10^2 Solar masses, then these could be detected by their microlensing effects on stars. So the MACHO and EROS projects were set up to survey tens of millions of stars in a nearby galaxy (the Large Magellanic Cloud; LMC). These measurements are photometric, not astrometric. The light from a star in the LMC is bent into two distorted images, but because these are separated by less than a milliarcsecond (100 times smaller than the resolution of the Hubble telescope) they cannot be seen individually. Instead astronomers measure how their combined brightness varies with time as the source and observer move relative to each other.

More than a decade later, the results of these experiments are still puzzling.

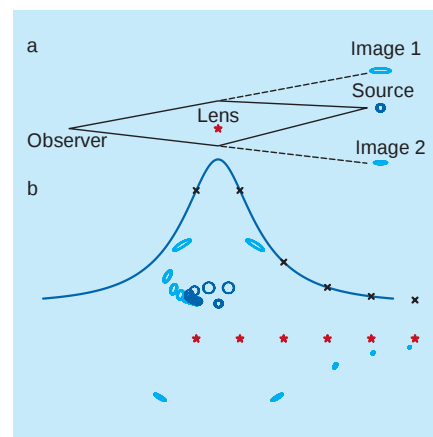


Figure 1 A step-by-step guide to gravitational microlensing. **a**, Light from the source star (green) is deflected by the lens (red), creating two enlarged and distorted images (cyan). **b**, As the lens passes by the source, the pair of images becomes first larger and then smaller, so that the observed light flux (blue curve) becomes brighter and then fainter — this is a photometric microlensing event. Alcock *et al.*³ have now obtained a direct image of a lens 6.3 years after the peak of the photometric light curve, thereby measuring the relative motion of the lens and source — this is the first astrometric microlensing measurement. The microlens images are much too small to resolve, but the centre of their combined light (blue circles) traces a small ellipse during the microlensing event. Future spacecraft will be able to measure this effect to determine the relative motion of a lens and source, and the lens mass, even if the lens emits no light.

MACHO found about 15 examples of microlensing in the direction of the LMC — only a fifth of the number needed to account for all the dark matter in the Milky Way, but five times too many to be explained by known populations of stars⁷ along the line of sight to the LMC. Frustratingly, most microlensing events yield absolutely no information about the distance to the lens, so it is not known whether it lies close to the source star in the LMC, close to the observer in our Galaxy, or in the vast space in between where we would expect to find MACHOs.

Now the MACHO team³ have succeeded in directly imaging one of their gravitational microlenses (LMC-5), and positively identify it as a star from the Milky Way. This not only provides a clue to the nature of these lenses, but is also the first successful merger of astrometric and photometric microlensing — a process that will eventually transform the field (see Fig. 1).

The original analysis⁸ of light from LMC-5 showed that it contained extra starlight from a faint red star, whose colour and brightness were consistent with it being in the Milky Way, rather than in the LMC, suggesting that this star might be the lens⁹. To test this idea, the MACHO collaboration³ obtained Hubble images of LMC-5, some six years after the original microlensing measurement. By this time the red star had moved 0.1 arcseconds away from the source, far enough to be seen as a separate object, but close enough to make its identification as the lens virtually certain.

By imaging the lens, the MACHO team have also made the first astrometric microlensing measurement. Because the source and lens were almost coincident at the time of the event, we can calculate their relative angular speed from their positions six years later. Further Hubble observations would allow astronomers to obtain their relative parallax, and so, according to Refsdal's original recipe, the lens mass. (MACHO have already reported³ a parallax measurement from a photometric observation of LMC-5, but this must still be considered tentative.)

Ultimately, the combination of photometry with astrometry will change the face of microlensing. With nearly microarcsecond precision, NASA's Space Interferometry Mission (SIM) will be able to measure the masses of about a dozen nearby stars with 1% accuracy¹⁰, in just the way that Refsdal originally proposed. But SIM will also combine photometric and astrometric microlensing to measure lens masses by another route, one that can be applied to dark as well as luminous matter. Even though SIM will not be able to see two separate images distorted by the lens, it can effectively determine their separation by tracking the motion of their combined light^{11,12}. Because SIM will

be in orbit around the Sun, its photometric observations will be affected by parallax relative to those obtained from the Earth (another seminal idea of Refsdal¹³). Combining SIM astrometry with Earth/SIM photometry will then yield the mass, distance and velocity of the lens, even if the lens is completely dark¹⁴.

This idea can be used not only to discover the nature of the lenses detected in the direction of the LMC, but also to measure the masses of a representative sample of all objects, dark and luminous, that inhabit our Milky Way. The OGLE collaboration¹⁵ is detecting hundreds of such lenses in the dense fields of stars found near the centre of the Milky Way, but almost nothing is known about their masses — at least for now. ■

Cell biology

Conservation signals location

Alexandre Costa and Paul Schedl

During development, many messenger RNAs are spread asymmetrically within cells. Surprisingly, in fruitflies the RNA signals and machinery used for distribution seem to be conserved in different developmental stages.

The asymmetric localization of messenger RNA molecules (mRNAs) is used in cells as diverse as eggs and neurons to restrict the expression and spatial distribution of proteins. mRNA localization depends both on special signals in the mRNAs themselves ('cis-acting' signals) and on the RNA-binding proteins that recognize these signals ('trans-acting' factors). The resulting RNA-protein complexes in turn interact with machinery that targets the mRNAs to a

Andrew P. Gould is in the Department of Astronomy, Ohio State University, Columbus, Ohio 43210-1106, USA.

e-mail: gould@astronomy.ohio-state.edu

1. Dyson, F. W., Eddington, A. S. & Davidson, C. *Phil. Trans. R. Soc. Lond. A* **220**, 291–333 (1920).
2. Refsdal, S. *Mon. Not. R. Astron. Soc.* **128**, 295–306 (1964).
3. Alcock, C. et al. *Nature* **414**, 617–619 (2001).
4. Alcock, C. et al. *Nature* **365**, 621–623 (1993).
5. Aubourg, E. et al. *Nature* **365**, 623–625 (1993).
6. Paczynski, B. *Astrophys. J.* **304**, 1–5 (1986).
7. Alcock, C. et al. *Astrophys. J.* **542**, 281–307 (2000).
8. Alcock, C. et al. *Astrophys. J.* **486**, 697–726 (1997).
9. Gould, A., Bahcall, J. N. & Flynn, C. *Astrophys. J.* **482**, 913–918 (1997).
10. Salim, S. & Gould, A. *Astrophys. J.* **539**, 241–257 (2000).
11. Paczynski, B. *Astrophys. J.* **494**, L23–L26 (1998).
12. Boden, A. F., Shao, M. & Van Buren, D. *Astrophys. J.* **502**, 538–549 (1998).
13. Refsdal, S. *Mon. Not. R. Astron. Soc.* **134**, 315–319 (1966).
14. Gould, A. & Salim, S. *Astrophys. J.* **524**, 794–804 (1999).
15. Wozniak, P. R. et al. *Acta Astron.* **51**, 175–219 (2001).

particular subcellular compartment, where they are ultimately translated into proteins. It has long been assumed that the signals and factors used for localization are unique to the cell type and the mRNA in question. But on page 611 of this issue Bullock and Ish-Horowicz¹ present evidence that, in fruitflies (*Drosophila melanogaster*), the localization of maternal mRNAs during egg development (oogenesis) and of mRNAs expressed during early embryo develop-

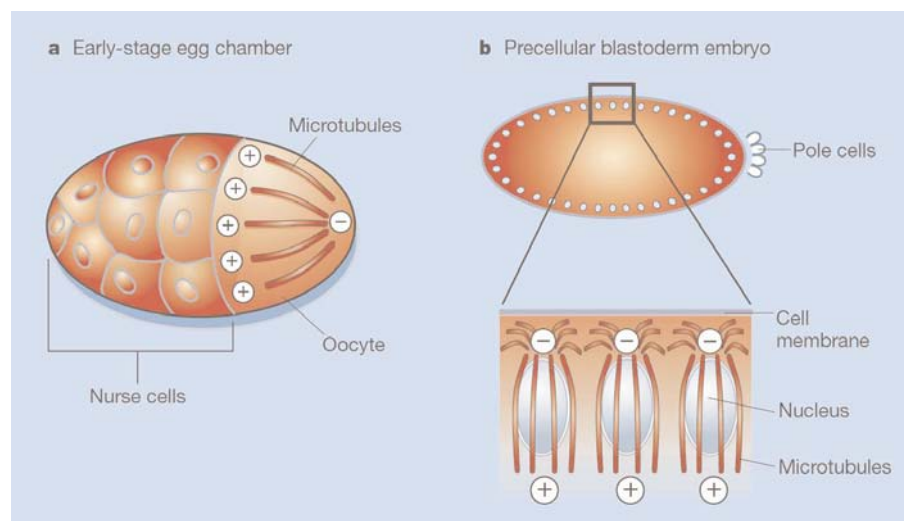


Figure 1 The asymmetric localization of mRNAs during oocyte and embryo development in *Drosophila*. **a**, An early-stage egg chamber. Several maternal mRNAs, synthesized in the nurse cells, are transported to the developing oocyte and accumulate at the 'minus' end of microtubules. **b**, A precellular blastoderm embryo. Nuclei aligned at the surface of the embryo synthesize pair-rule mRNAs, which accumulate apically after being transported towards the microtubule minus end.



100 YEARS AGO

For some time past there has been a large and apparently influential party of alarmists with regard to the use of preservatives [in food]. These have all been heard in length by the Committee which has just reported. Their evidence consisted for the most part of elaborate *a priori* argument, in support of which the most profound erudition was occasionally produced; but, as the report politely says, the opinion expressed was not always based directly on fact. In fact, if an inquirer turns the 500 pages of the Blue-book over in search of unequivocal instances of injury to health from preservatives or, indeed, colouring matters in food he will be lucky if he finds a single one... Upon such data it is obvious that the prohibition of preservatives *en masse* was out of the question, and the recommendations of the Committee practically resolve themselves into the regulation and control rather than the prohibition of preservatives. There are, however, two exceptions to this; formaline or formic aldehyde is prohibited altogether, and all preservatives and colouring matters are prohibited in milk.

From *Nature* 5 December 1901.

50 YEARS AGO

It has been suggested by Kistiakovsky that the mechanism of smell could be explained by the existence in the olfactory mucosa of four enzyme groups... and that odoriferous substances are distinguished by their differential inhibition of particular enzyme groups. The same explanation might be used to account for a mechanism capable of distinguishing between substances of different taste... we suggest the following tentative hypothesis to explain [the enzymes'] relation to the mechanism of taste. The chemical reaction involved in the breakdown of substrate by enzyme presumably gives rise to ionic changes which would induce currents in nearby nerve fibres... A tasting substance coming into contact with superficial enzyme sites... would inhibit the activity of an enzyme in one site, accelerate the activity of an enzyme in another site and have no effect on enzymes in other sites and so on. It would therefore alter the pattern of nerve impulses reaching the brain, and each substance would produce its own individual pattern. Since there are six main sites of enzyme activity... we have a mechanism for distinguishing between 2,160 tasting substances.

From *Nature* 8 December 1951.

ment depends on common signals and factors.

Asymmetric mRNA localization is crucial in establishing polarity in developing *Drosophila* eggs and early embryos. During egg (oocyte) development, mRNAs expressed in a cluster of interconnected auxiliary cells (nurse cells) are first transported into the oocyte and then targeted to specific locations within the oocyte (Fig. 1a). Once there, some of these maternal mRNAs are translated more or less immediately; their protein products help to lay down the oocyte's two 'polarity axes' (anterior-posterior and dorsal-ventral). Other localized maternal mRNAs, such as *bicoid*, are translated in the early embryo rather than the oocyte, producing a gradient of bicoid protein. This gradient helps to establish embryonic polarity by directing the initial expression of embryonic patterning genes in 'precellular blastoderm embryos' — an early stage of development in which thousands of cell nuclei are distributed around the surface of the embryo and share a common cytoplasm (Fig. 1b).

The first such patterning genes to be expressed are the gap genes, and their products form short-range gradients that subdivide the embryo into broad domains. The gap mRNAs are distributed uniformly in the cytoplasm at the surface of the embryo, and this is thought to facilitate the formation of short-range gradients extending across several nuclei². The gap proteins in turn control the expression of the pair-rule genes, which further subdivide the embryo. In contrast with the unlocalized gap mRNAs, pair-rule mRNAs are targeted apically, that is, to the cytoplasm just above the nuclei that express the mRNAs³. This decreases the lateral diffusion of the encoded proteins, helping to refine the pattern of pair-rule gene expression and to promote the subdivision of the embryo into segments once cells have formed.

There are hints that some of the steps involved in localizing maternal mRNAs in oocytes and pair-rule mRNAs in early embryos depend on common mechanisms. First, an analysis of mRNA localization in early embryos that lack large sections of their genetic material indicated that the *trans*-acting factors required for apical localization must be maternally derived rather than expressed in the embryo⁴. Second, pair-rule mRNAs are transported to the apical cytoplasm along tracks known as microtubules, by motor proteins that move towards the 'minus' end of microtubules⁵. Similarly, the localization of several maternal mRNAs in oocytes depends on microtubules, and accumulation is directed towards the minus end⁶.

Bullock and Ish-Horowicz¹ test the idea directly by investigating whether the localization machinery in blastoderm embryos

can target maternal mRNAs (rather than the usual pair-rule mRNAs) to an apical location. They find that maternal messages such as *K10*, *gurken*, *bicoid*, *oskar* and *nanos* are recognized by the embryonic machinery and localized preferentially to the apical cytoplasm. Conversely, pair-rule mRNAs that are expressed artificially during oogenesis accumulate in the oocyte in a pattern resembling that of *K10* and several other maternal mRNAs.

Two other lines of evidence indicate that at least some of the same *trans*-acting factors must be used during oogenesis and embryo development. The first comes from the authors' studies¹ of two proteins, Bicaudal D and Egalitarian, which are required for mRNA localization during egg development and form a complex that accumulates at the minus ends of microtubules in oocytes^{7,8}. Bullock and Ish-Horowicz¹ find that these proteins are also enriched apically in embryos and localize with a component of a minus-end-directed motor protein, dynein. Moreover, both proteins are required for the apical localization of pair-rule (naturally expressed) and maternal (artificially expressed) mRNAs in early embryos.

Second, Bullock and Ish-Horowicz¹ studied *cis*-acting localization signals. They show that the shortest RNA signal known to be active during egg development, the *K10TSL*, can also target normally unlocalized mRNAs to an apical location in embryos. This RNA signal is predicted to fold into a shape known as a 'stem-loop' structure. Mutations that disrupt this structure block the localization of *K10* during egg development; compensatory mutations that restore the structure also restore localization⁹. Bullock and Ish-Horowicz¹ find that these disrupting and compensatory mutations have the same effects on mRNA targeting in embryos, arguing that the factors that recognize the stem-loop in eggs are likely to be present in embryos, too. Conversely, pair-rule transcripts that lack the minimum apical-localization element fail to accumulate in the oocyte when expressed during oogenesis.

The simplest explanation is that the same signals and factors are used to recognize and localize different mRNAs in different developmental contexts. If true, one might expect that mRNAs that show similar localization patterns in oocytes or embryos would have similar signals. Indeed, the *orb* mRNA, which is localized similarly to *K10* in oocytes, contains a localization element that is closely related in sequence and structure to the *K10TSL*⁹. Moreover, a 150-nucleotide element involved in targeting *oskar* mRNA during oogenesis¹⁰ has a stem-loop structure resembling that in the *K10TSL*, and the *K10TSL* can substitute for this element⁹.

However, *orb* (and *oskar*) may be the exception rather than the rule: the localiza-

tion elements in other maternal mRNAs and in pair-rule mRNAs seem to bear little sequence or structural similarity to the *K10TLS*. So an alternative model is that several different types of signal are used to mark mRNAs that accumulate in the oocyte or are localized apically in embryos. Distinct RNA-binding proteins would recognize these different signals and couple the mRNAs to the same basic localization machinery (whose components would include Bicardal D and Egalitarian). Finally, although the initial oocyte localization of maternal RNAs such as *K10* and *oskar* seems to proceed along a common pathway, there must be steps and mechanisms that are unique to at least a subset of these mRNAs, as the ultimate destina-

tions of maternal RNAs within the oocyte can be quite different. ■

Alexandre Costa and Paul Schedl are in the Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, USA. e-mail: pschedl@molbio.princeton.edu

1. Bullock, S. L. & Ish-Horowicz, D. *Nature* **414**, 611–616 (2001).
2. Hülskamp, M., Pfeffe, C. & Tautz, D. *Nature* **346**, 577–580 (1990).
3. Davis, I. & Ish-Horowicz, D. *Cell* **67**, 927–940 (1991).
4. Francis-Lang, H., Davis, I. & Ish-Horowicz, D. *EMBO J.* **15**, 640–649 (1996).
5. Wilkie, G. S. & Davis, I. *Cell* **105**, 209–219 (2001).
6. Lasko, P. *FASEB J.* **13**, 421–433 (1999).
7. Mach, M. M. & Lehmann, R. *Genes Dev.* **11**, 423–435 (1997).
8. Suter, B. & Steward, R. *Cell* **67**, 917–926 (1991).
9. Serano, T. L. & Cohen, R. S. *Development* **121**, 3809–3818 (1995).
10. Kim-Ha, J., Webster, P. J., Smith, J. L. & MacDonald, P. *Development* **119**, 169–178 (1993).

Human evolution

Questions of growth

Jacopo Moggi-Cecchi

The evolution of an extended childhood had implications for human society and culture. New analyses of dental development in fossil hominins suggest that our lengthy growth processes arose quite late in evolution.

Humans are unique among primates. Some of our unusual characteristics — our relatively large brains and the way we move, for example — are readily apparent. Others are less so, such as our long lifespan, delayed reproduction and prolonged childhood¹. These 'life-history' features do not leave direct traces in the fossil record, so it is incredibly difficult to figure out when and how they evolved. In an effort to find out, palaeoanthropologists have turned to teeth. In primates, there is a correlation between some life-history variables and features of dental development². Moreover, the eruption of certain teeth acts as a marker of growth; for example, the appearance of the third permanent molar — the

wisdom tooth — marks the end of the juvenile period. So studies of tooth development in fossil hominins should provide information about growth processes and, by extension, the origin of our prolonged childhood. On page 628 of this issue, Dean and colleagues³ analyse dental development in a wide range of extinct hominin species, with intriguing results.

Hominins include modern humans, as well as fossil species that are more closely related to humans than to chimpanzees — in other words, the genus *Homo* and the australopiths (*Ardipithecus*, *Australopithecus*, *Paranthropus* and *Kenyanthropus*)⁴. One approach to studying dental development requires fossils of young hominins⁵. These

can be studied either directly or with computed tomography scans⁶, revealing the pattern of tooth development. Results from this method have been used to suggest that *Homo habilis* (which dates to some 2.3 million to 1.8 million years ago) had a pattern of tooth development similar to that of the more primitive *Australopithecus*, whereas *H. erectus*/*H. ergaster* (1.9 million to 0.8 million years ago) shares similarities with modern humans⁷.

A second kind of analysis provides information on the rate, rather than pattern, of tooth development. This approach is based on the fact that the structure of a tooth preserves a record of development, mainly in the enamel (found on the crowns of the teeth) and the dentine (the internal hard tissue in a tooth)^{8,9}. The inner enamel in particular shows incremental markings (cross-striations), seen in naturally fractured or experimentally sectioned teeth, that occur during the daily deposition of enamel by ameloblast cells (Fig. 1). The number of cross-striations per millimetre provides an estimate of the rate of enamel formation. Another form of periodic marking, known as the striae of Retzius, is used to examine crown formation. Counting the associated surface features (perikymata) provides estimates of the duration of crown formation.

Dean *et al.*³ applied this second kind of analysis to a wide range of fossil hominin species, including *Australopithecus anamensis* (which dates to 4.2 million to 3.9 million years ago), Neanderthals (300,000 to 28,000 years ago), and the ape *Proconsul* (18 million years ago). The study covers complete specimens — as in the teeth of the famous 'Turkana boy', an almost complete specimen attributed to *H. ergaster*¹⁰ — and isolated teeth and even fragments of a single tooth. The evidence indicates that, in terms of enamel-formation rates and crown-formation times, australopiths and early members of the genus *Homo* (*H. habilis* and *H. erectus*) resemble modern and fossil apes more closely than they do modern humans.

The results confirm the similarities of *H. habilis* and *Australopithecus*^{7,11}. More intriguing, however, are the implications for interpreting the life history of *H. erectus*. Dean *et al.*³ propose that the developmental processes in *H. erectus* differ only slightly from those of earlier hominins (australopiths). This proposal is in agreement with the view that *H. erectus*/*H. ergaster* is only slightly different from australopiths in its relative brain size^{11,12} (there is suggested to be a relationship between an increase in brain size and extension of the postnatal period of development^{12,13}). However, it has been argued that *H. erectus*/*H. ergaster* resembled modern humans in terms of its posture, body size and limb proportions¹¹. So it seems that the human brain and body structures evolved in a mosaic pattern, with human-

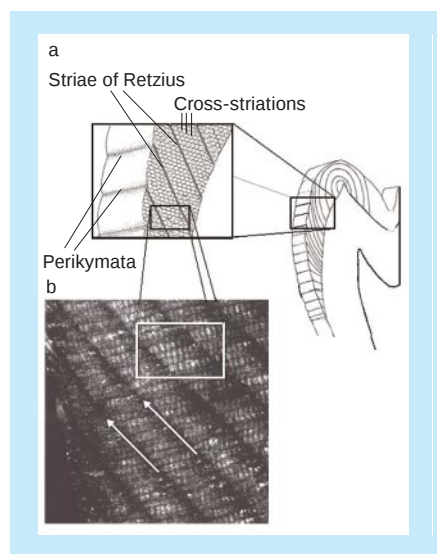


Figure 1 Dental microstructure. a, A diagram, and b, a micrograph showing the incremental markings in tooth enamel. Cross-striations occur daily as ameloblast cells secrete enamel. The rate of enamel formation can be estimated by counting the number of cross-striations per millimetre. Perikymata, seen on the external surface of the teeth, are associated with the striae of Retzius (large arrows in b). They provide estimates of the duration of crown formation, as they have a modal periodicity of nine days in apes and humans.

like rates of dental development and a marked increase in relative brain size apparently occurring later than other human-like body structures. The slow rate of enamel growth that is typical of modern humans, and is associated with an extended growth period, is first seen with the large-brained Neanderthals.

What about hominin species intermediate in time between *H. erectus* and Neanderthals? On the basis of dental developmental patterns, it has been suggested that 0.8-million-year-old hominin remains from Spain (attributed to the species *H. antecessor*) had a developmental profile like that of modern humans¹⁴. But Dean *et al.*³ warn us that this does not necessarily imply human-like rates of growth. Because dental developmental patterns^{7,14} tell only part of the story, studying the rates of tooth development in Middle Pleistocene hominins should be revealing.

A cautionary note, however, is that when dental developmental data are not the only source of information on the growth process, different lines of evidence may lead to different conclusions. For example, combined estimates of the age at death of the Turkana boy — based on analysis of dental and skeletal development and on reconstruction of the stature of this individual — have suggested that his overall pattern of growth was within the normal range of variation seen in modern humans¹⁵.

Finally, the results of Dean *et al.*'s study³ have other implications. First, they show that the thick enamel of modern human teeth may not be homologous to the thick enamel of early hominins, being the result of a different developmental process. This finding underlines the importance of incorporating information on developmental mechanisms when interpreting the significance of certain traits in analysing evolutionary relationships¹⁶. Second, the new results support the idea that specific morphological characters (such as brain size, enamel thickness or bipedalism), as observed or inferred in fossil specimens, cannot be interpreted in isolation to indicate affinities with modern humanity. It is becoming increasingly clear that many features thought to be typical of modern humans may have evolved more than once. Dean *et al.*'s analysis raises new challenges in the search for fossil evidence of those characteristics that define both our genus¹¹ and our species. ■

Jacopo Moggi-Cecchi is in the Laboratori di Antropologia, Dipartimento di Biologia Animale e Genetica, Università di Firenze, via del Proconsolo, 12, Florence 50122, Italy.
e-mail: jacopo@unifi.it

1. Harvey, P. H. & Clutton-Brock, T. H. *Evolution* **39**, 559–581 (1985).
2. Smith, B. H. *Evolution* **43**, 683–688 (1989).
3. Dean, C. *et al.* *Nature* **414**, 628–631 (2001).
4. Wood, B. A. & Richmond, B. G. *J. Anat.* **196**, 19–60 (2000).
5. Smith, B. H. *Am. J. Phys. Anthr.* **94**, 307–325 (1994).

6. Conroy, G. C. & Vannier, M. W. *Nature* **329**, 625–627 (1987).
7. Moggi-Cecchi, J. in *Humanity from African Naissance to Coming Millennia* (eds Tobias, P. V., Raath, M. A., Moggi-Cecchi, J. & Doyle, G. A.) 125–134 (Firenze Univ. Press, Florence, & Witwatersrand Univ. Press, Johannesburg, 2001).
8. Beynon, A. D. & Dean, M. C. *Nature* **335**, 509–514 (1988).
9. Dean, C. *J. Anat.* **197**, 77–101 (2000).
10. Brown, F. H., Harris, J. M., Leakey, R. E. & Walker, A. C. *Nature* **316**, 788–792 (1985).

11. Wood, B. A. & Collard, M. *Science* **284**, 65–71 (1999).
12. Smith, B. H. & Tompkins, R. L. *Annu. Rev. Anthropol.* **24**, 257–279 (1995).
13. Martin, R. D. *Human Brain Evolution in an Ecological Context: 52nd James Arthur Lecture on the Evolution of the Human Brain* (Am. Mus. Nat. Hist., New York, 1983).
14. Bermúdez de Castro, J. M. *et al.* *Proc. Natl Acad. Sci. USA* **96**, 4210–4213 (1999).
15. Clegg, M. & Aiello, L. C. *Am. J. Phys. Anthr.* **110**, 81–94 (1999).
16. McCollum, M. A. & Sharpe, P. T. *BioEssays* **23**, 481–493 (2001).

Cell biology

High-performance fungal motors

Susan P. Gilbert

Kinesin proteins are nanoscale transport vehicles. Analysis of the structure and function of one such protein has revealed the secrets of its rapid movement.

Motor proteins are tiny vehicles that move molecular cargoes around inside cells. These minute cellular machines come in three broad families, the kinesins, the myosins and the dyneins. There are over 250 kinesin-like proteins, and they are involved in processes as diverse as the movement of chromosomes and the dynamics of cell membranes. They all have a similar catalytic portion, known as the motor domain, but beyond this they are astonishingly varied — in their location within cells, their structural organization, and the movement they generate¹. For instance, the 'conventional' kinesin from the fungus *Neurospora crassa* is incredibly quick, moving along filamentous tracks called microtubules at speeds of 2.5 μm per second — some five times faster than other conventional kinesins. Writing in the *EMBO Journal*, Kallipolitou and colleagues² and Song and co-workers³ suggest how such speeds can be achieved.

Conventional kinesins are dimeric — they consist of two identical proteins, each of which has a motor domain (the 'head') and a cargo-binding domain, with a stalk in between. The two stalks coil around each other to produce the dimer. The two heads can attach to a microtubule track, and cooperate so that kinesin can 'walk' for several hundred steps without detaching. Each 8-nanometre step is coupled to the hydrolysis of one molecule of the nucleotide ATP^{4,5} (the cellular energy store) to produce ADP and inorganic phosphate, which are then released so that the head can bind another ATP. This type of movement is known as 'processive'. It is achieved^{6–8} by a mechanism that keeps the heads out of sync with each other, preventing them from being in the same nucleotide-binding state at the same time and so detaching simultaneously from the microtubule. Another feature of conventional kinesins is that they move in only one direction along microtubules.

How do such processivity and direction-

ality come about? Two structural elements of the protein — the neck linker and the neck — appear to be crucial^{9,10}. The neck linker lies just outside the catalytic core of the head, and

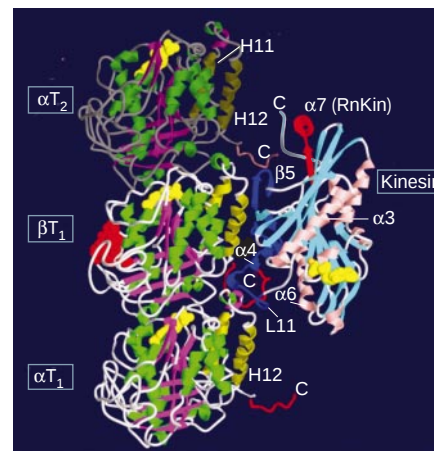


Figure 1 Model of a microtubule filament (left) with the bound motor domain of *Neurospora crassa* conventional kinesin, based on Song *et al.*³. See also the movie at ref. 18. In the microtubule, two α -tubulin subunits (αT_1 and αT_2) and a β -tubulin (βT_1) are shown (structure adapted from ref. 19). Kinesin would move along the microtubule towards the top of the figure. *N. crassa* kinesin and the microtubule interact predominantly through helices H11 and H12 (mustard yellow coils) and the carboxy-terminal tail (thin red strand, C) of βT_1 , and the region of kinesin that includes the structural elements in dark blue ($\alpha 4$ –L12– $\alpha 5$ and $\beta 5a$ –L8). Other interactions may include the carboxy-terminal tail (pink, C) of αT_2 and kinesin's neck linker/neck region (grey), as well as αT_1 and kinesin's L11. The top of the kinesin core shows the neck domain in two conformations: a random coil found in NcKin (protein backbone in grey), and the ordered conformation of $\alpha 7$ from rat brain kinesin (RnKin)¹³ in red. The group of yellow spheres represents GTP and GDP in tubulin and ADP in NcKin. The drug taxol (red spheres) is shown bound to βT_1 .

the neck leads from the neck linker to the coiled stalk. The binding of ATP induces a series of conformational changes that position the stepping head forwards¹¹; in particular, ATP-driven docking of the neck linker onto the core of the attached head has been suggested to influence the direction of movement and processivity^{9,12}. Other models for kinesin stepping include a role for partial unwinding and subsequent recoiling of the neck or coiled stalks.

What about the *N. crassa* conventional kinesin (NcKin)? This is the Ferrari of the kinesin motor family, so its structure and mechanisms might be expected to differ somewhat from its more sluggish relatives. Kallipolitou *et al.*² and Song *et al.*³ provide, respectively, biochemical and structural clues to NcKin's souped-up performance.

One obvious structural feature³, which contrasts markedly with available structures of rat^{13,14} and human¹⁵ conventional kinesins, is that NcKin's neck linker/neck domain appears as a random coil, implying that it is highly mobile (Fig. 1). Furthermore, Kallipolitou *et al.*'s biochemical analyses² reveal that artificial NcKin motors that include the catalytic core, the neck linker and the neck fail to dimerize, in contrast to similar constructs of fruitfly kinesin. The neck linker, neck and part of the adjacent hinge domain are all required for NcKin's dimerization, rapid motility and processivity². It seems, then, that NcKin's neck domain has unusual properties. So, too, does its head domain.

Kinesins, myosins and molecular switches known as G proteins, although unrelated in amino-acid sequence, share remarkable structural similarity in their nucleotide-binding pockets, suggesting that they have a similar mechanism of nucleotide hydrolysis^{10,12}. The nucleotide-sensing regions are formed by three structural elements, known as the P-loop, switch I and switch II. During nucleotide binding and hydrolysis, the switch regions are mobile, and small movements trigger a cascade of structural alterations in the rest of the protein. As Song *et al.*³ find, the biggest differences between the *N. crassa* and rat conventional kinesins are in regions implicated in these structural changes — that is, in switch I, switch II and the microtubule-binding surface.

These differences mean that NcKin has a nucleotide-binding pocket that is much more open than that of other conventional kinesins. In particular, loop 9 in the switch I region bends out, making the pocket very wide. Moreover, the nucleotide-dependent displacement of switch I resembles what happens when the G protein Ras binds a factor that accelerates its nucleotide exchange. This suggests that microtubules — which activate ADP release in kinesins — might further accelerate the exchange of ADP for

ATP in NcKin. Comparison of NcKin with several other kinesins reveals a direct correlation between the rate of ATP turnover, the speed of the motor, and the openness of the nucleotide-binding pocket^{2,3}. The structure of this pocket in NcKin suggests that this motor's fast movement is due in part to rapid ATP turnover, including accelerated ADP/ATP exchange.

NcKin has another unique structural feature that might affect the rate of ATP turnover³: it has contacts with the microtubule surface that are not seen in other kinesin structures (Fig. 1). Microtubules consist of long chains of alternating α -tubulin and β -tubulin subunits, and most of the contacts with a conventional kinesin involve β -tubulin's carboxy-terminal part and three structural features of kinesin's switch II region¹⁶. In NcKin³, a further structural feature from switch II — loop 11 — also contacts α -tubulin. Loop 11 is also part of the switch II region that forms the nucleotide-binding pocket, so its interaction with α -tubulin probably affects ATP hydrolysis.

Part of NcKin's mobile neck also interacts with microtubules³, in a way that could explain the increase in processivity seen¹⁷ when extra positive charge is added to the neck, increasing its interaction with tubulin's negatively charged carboxy terminus. Kallipolitou *et al.* suggest² that the mobile neck of NcKin may enhance both processivity and rate of movement.

It seems, then, that the high performance of NcKin stems from its unusually wide ATP-binding pocket, its mobile neck and its extra interactions with microtubules. These interpretations will no doubt trigger some dissent. But they should lead researchers to test the effect of a more flexible neck and more open nucleotide-binding pocket on processivity, the rate of ATP turnover and the speed of these splendid molecular machines. ■

Susan P. Gilbert is in the Department of Biological Sciences, 518 Langley Hall, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, USA.

e-mail: spg1@pitt.edu

1. <http://www.blocks.fhrc.org/~kinesin>
2. Kallipolitou, A. *et al.* *EMBO J.* **20**, 6226–6235 (2001).
3. Song, Y.-H. *et al.* *EMBO J.* **20**, 6213–6225 (2001).
4. Schnitzer, M. J. & Block, S. M. *Nature* **388**, 386–390 (1997).
5. Hua, W. *et al.* *Nature* **388**, 390–393 (1997).
6. Hackney, D. D. *Proc. Natl Acad. Sci. USA* **91**, 6865–6869 (1994).
7. Ma, Y. Z. & Taylor, E. W. *J. Biol. Chem.* **272**, 724–730 (1997).
8. Gilbert, S. P., Moyer, M. L. & Johnson, K. A. *Biochemistry* **37**, 792–799 (1998).
9. Rice, S. *et al.* *Nature* **402**, 778–784 (1999).
10. Vale, R. D. & Milligan, R. A. *Science* **288**, 88–95 (2000).
11. Schnitzer, M. J., Visscher, K. & Block, S. M. *Nature Cell Biol.* **2**, 718–723 (2000).
12. Kikkawa, M. *et al.* *Nature* **411**, 439–445 (2001).
13. Sack, S. *et al.* *Biochemistry* **36**, 16155–16165 (1997).
14. Kozielski, F. *et al.* *Cell* **91**, 985–994 (1997).
15. Kull, F. J. *et al.* *Nature* **380**, 550–555 (1996).
16. Hoenger, A. *et al.* *J. Mol. Biol.* **297**, 1087–1103 (2000).
17. Thorn, K. S., Ubersax, J. A. & Vale, R. D. *J. Cell Biol.* **151**, 1093–1100 (2000).
18. <http://www.mpasmb-hamburg.mpg.de/ktdock>
19. Nogales, E. *et al.* *Cell* **96**, 79–88 (1999).

Daedalus

Extra security

Airline security is something we must all face. Many engineers are making clever scanners and sniffers to check our luggage. But Daedalus wants to reduce the amount of stuff we take in the first place. One proposed scheme reduces the nonsense of 'duty free' purchases. Payment at the departure airport leads to delivery at the destination. Airlines no longer have to carry (and scan) a vast amount of impure alcohol in 'duty free' flight bags. So Daedalus is extending this idea. DREADCO security guards are now examining the bulk luggage to spot common objects that can easily be specified and replicated at the other end.

The main problem is clothing. Casually dressed flyers often carry a smart suit or outfit in their luggage. In principle, the details could be faxed to the other end. The clothing industry has largely perfected the reduction of any suit to a set of numbers, but fashion sadly complicates the choice. Colour, pattern, cut and other subtle variables greatly multiply the items that would need to be held at the receiving airport. DREADCO does not want to take on the fashion industry, but he hopes to nudge it in a socially useful direction. Casual flightwear might be challenged: with luck a standard 'airline suit' would identify the frequent flier to fashionable advantage. A flight-resistant suit or outfit in some old 'wonder fibre' could be revived for this job. Casual flight-creased clothes might become fashionable themselves, but Daedalus admits this is out of his hands.

Another item studied by the security guards is the laptop computer. Many flyers would be glad not to carry this heavy and expensive toy. Even the most ardent key-basher is unlikely to fill a memory insert during a flight. With a standard aircraft computer (including a power supply, like the standard audio system), patrons could insert their memory device for the flight, do their work, and rely on the digital environment at the other end. Indeed, the next generation of portable computers should be designed so that changeable memory can be divorced from keyboard, screen and such standard items. Laptop owners would be pleased to take a mere memory everywhere, and use it to update their machine when they get home.

Other items frequently carried include pens, paper, calculators, cosmetics and toiletries. A standard 'travel pack' could be provided at the destination airport. The saving on security, scanners, and needless carriage should reduce the unavoidable costs of air travel.

David Jones

Plastic transistors in active-matrix displays

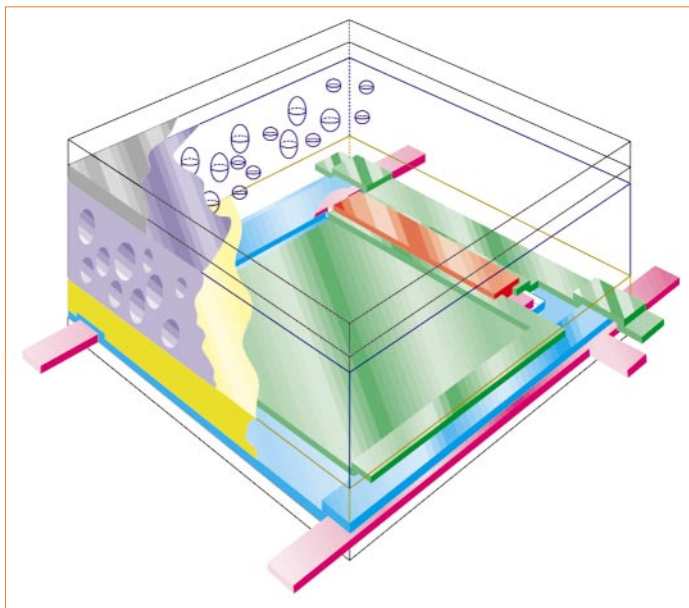
The handling of grey levels by these large displays paves the way for electronic paper.

The main advantages of using soluble semiconductive polymers in micro-electronic devices are ease of processing^{1–3} and mechanical flexibility⁴. Here we describe an active-matrix display with 64×64 pixels, each driven by a thin-film transistor with a solution-processed polymer semiconductor. In a significant step towards low-cost flexible displays, this polymer-dispersed liquid-crystal arrangement gives a reflective, low-power display with paper-like contrast, which can handle 256 grey levels while being refreshed at video speed.

The pixel layout of the 2-inch (5.1-cm) display, which is based on the design for a small hand-held display, is shown in Fig. 1. First, the row electrodes are created by depositing and structuring a gold layer on a glass substrate. Next, an insulating layer of silicon nitride is applied. A second, patterned gold layer forms the column electrodes and the pixel pad. The pixel pad acts as an in-cell reflector, occupying 83% of the display area. On top of this stack, a polythiénylenevinylene precursor film is spin-coated, converted into a semiconductor⁵ and patterned by photolithography. A spin-coated polyvinylphenol layer protects the active matrix.

Spacers are sprayed on top of this active matrix. A second substrate, coated with indium tin oxide, is applied over the spacers and glued to the active matrix. Finally, the display is filled with polymer-dispersed liquid crystals⁶, thereby avoiding polarizers and an alignment layer. This technology yields a low-cost display with a high contrast and a wide viewing angle⁷, and even allows flexible displays to be produced^{8,9}. A polymer-dispersed liquid-crystal

Figure 1 Layout of one pixel ($540 \times 540 \text{ nm}^2$) of an active-matrix display driven by transistors with a polymer semiconductor. The layout consists of the following layers: the row electrode (the gate; pink), insulator layer (blue), column electrode and pixel pad (the source and drain; green), semiconductor (red), protective layer (yellow), polymer-dispersed liquid-crystal layer (violet) and common electrode (grey), all sandwiched between two glass substrates (transparent). The length of the thin-film transistor (TFT) channel is 5 μm ; the width is 400 nm .



pixel is switched between light-scattering and transparent states by applying a voltage between the electrical contacts.

The display is driven line-by-line. During one frame time, all rows are sequentially selected by applying a negative voltage that reduces the channel resistance of its thin-film transistors (TFTs). The column voltage is then transferred through the channel of the TFTs to the corresponding pixels. During the rest of the frame time, the channel resistance is high, thereby retaining the charge on the pixels while the other rows are addressed.

An image on the display is shown in Fig. 2. It contains 256 grey levels, as required for displaying natural scenes. The maximum contrast ratio is 8.6, which is comparable to that of black ink on paper. The columns are driven between -12 V and 12 V to switch the pixels. The rows, which are connected to the gate of the TFTs, are driven between -27 V and 27 V . The maximum on-current of the TFT is $0.8 \mu\text{A}$; an average of $0.4 \mu\text{A}$ is sufficient to charge a pixel during the row-selection time. The field-effect mobility is $1.5 \times 10^{-3} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. The off-current is below 20 pA , low enough to retain at least 99% of the pixel charge during the rest of the frame time. The on/off current ratio is 4×10^4 , which matches the specifications for a display of this size. Because of the optimal pixel design, the display can be driven at refresh rates of up to 100 Hz.

Well defined grey levels require sufficient control of the 'spread' on the TFT parameters such as mobility, leakage

current and threshold voltage. Measurements show a standard deviation in the contrast of a typical line in the display of 0.11 for white pixels and 0.14 for pixels switched to mid-grey. This small difference indicates that the spread on TFT parameters is only a minor factor in the uniformity of this display. We intend to optimize our solution-based technology to obtain the same uniformity over an even larger area, paving the way for a low-cost production process for large sheets of electronic paper.

H. E. A. Huitema*, G. H. Gelinck*, J. B. P. H. van der Putten*, K. E. Kuijk*, C. M. Hart*, E. Cantatore*, P. T. Herwig†, A. J. J. M. van Breemen†, D. M. de Leeuw*

*Philips Research, Professor Holstlaan 4, 5656 AA Eindhoven, The Netherlands
e-mail: edzer.huitema@philips.com

†Materials Technology Division, Netherlands Organisation for Applied Scientific Research, Institute of Industrial Technology, PO Box 6235, 5600 HE Eindhoven, The Netherlands

1. Gelinck, G. H., Geuns, T. C. T. & de Leeuw, D. M. *Appl. Phys. Lett.* **77**, 1487–1489 (2000).
2. Sirringhaus, H., Tessler, N. & Friend, R. H. *Science* **280**, 1741–1744 (1998).
3. Levi, B. G. *Phys. Today* 20–22 (February 2001).
4. Voss, D. *Nature* **407**, 442–444 (2000).
5. Tokito, S., Momii, T., Murata, H., Tsutsui, T. & Saito, S. *Polymer* **31**, 1137–1141 (1990).
6. Vaz, N. A., Smith, G. W. & Montgomery, G. P. Jr *Liquid Cryst.* **146**, 1–15 (1987).
7. Cornelissen, H. J., Neijzen, J. H. M., Paulissen, F. A. M. A. & Schlangen, L. J. M. *J. Soc. Inform. Display* **7**, 37–41 (1999).
8. Kane, M. G. et al. *IEEE Electr. Dev. Lett.* **21**, 534–536 (2000).
9. Rogers, J. A. et al. *Proc. Natl Acad. Sci. USA* **98**, 4835–4840 (2001).

Competing financial interests: declared none.

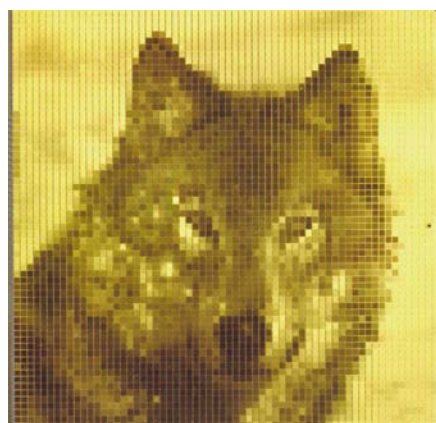


Figure 2 An image on the multipixel display driven by 4,096 thin-film transistors, with a solution-processed polythiénylenevinylene semiconductor. The image contains 256 grey levels; the display is refreshed at 50 Hz.

Phenology

False estimates of the advance of spring

Bias is introduced into almost all recent reports of climate-related trends in the phenology of spring events (for example, the timing of migration, egg laying and ice melt) by giving the calendar date of such occurrences each year, rather than their timing relative to the vernal equinox (refs 1–8, but see ref. 9). Most of these studies overestimate the advance of spring events, as the calendar date of the vernal equinox shows a trend to become earlier throughout any century, although this bias is small in the examples published so far. However, its magnitude cannot be predicted for any data set that is extended into the twenty-first century, because of long-term changes in the date of the vernal equinox. As phenological data are important for studying climate change, trends need to be reported in terms that accurately reflect changes to the Earth system.

Trends in the calendar date of the vernal equinox within centuries arise from the discrepancy between our average 365.25-day calendar (accounting for leap years) and the roughly 365.2422-day 'vernal equinox year' (the average time between successive vernal equinoxes). This difference causes the equinox to arrive about 0.78 days earlier on the calendar over 100 years (Fig. 1a). Because years that are divisible by 100 (but not by 400) are not leap years¹⁰, trends

towards earlier vernal equinoxes are broken at the beginning of most centuries and the equinox is 'reset' to a later date (Fig. 1b). This means that a given calendar date in spring will occur further from the vernal equinox towards the end of any century. As a result, trends towards earlier spring will tend to be overestimated.

The magnitude of this bias in reported phenological trends will depend on the length of the record, the period it covers, and the actual scatter of the data. As the magnitude of the bias cannot generally be predicted, I converted published data on examples of phenological events to express the time elapsed since the vernal equinox for each year of the data set, and compared linear trends between the original and transformed data sets.

My analysis of these examples showed biases of up to 10% in the originally reported trends (see supplementary information). Trends towards earlier spring are still significant, although generally smaller than the original data would indicate. Data sets that span the twentieth century show the largest bias. Although shorter-term data sets from the late twentieth century (see, for example, refs 1, 5, 7, 8) have smaller biases (1–5%), their biases will increase if they are extended into the twenty-first century for two reasons. First, the discrepancy between trends based on calendar dates and those based on proximity to the vernal equinox will continue to grow in the twenty-first century because the spring equinox will continue to become earlier until 2100 (Fig. 1b). Second, if the rate of global warming increases as expected and phenological trends follow accordingly, trends towards earlier spring in these data sets will be driven by the time period when the difference between calendar date and the date of vernal equinox is greatest.

Two data sets that span two previous centuries or longer² indicate that the advancement of spring may have been underestimated in some cases. This is probably an effect of unequal bias magnitude in data from successive centuries.

Although any underestimation of the delay of autumn events might be expected to offset bias in spring timing, most published phenological studies analyse spring events only. Reporting phenological events in relation to the vernal equinox is a simple procedure that does not require any change in data-collection methods. Phenological information is becoming more important for studying climate change because of the increased availability of long-term records¹¹, new methods for reconciling traditional records with recent remotely sensed data, and the incorporation of phenology into geographical-scale models¹². It is therefore essential that we

report phenological trends in terms that accurately reflect changes to the Earth system.

Raphael Sagarin

Hopkins Marine Station, Pacific Grove, California 93950, USA

e-mail: sagarin@stanford.edu

1. Inouye, D. W., Barr, B., Armitage, K. B. & Inouye, B. D. *Proc. Natl Acad. Sci. USA* **97**, 1630–1633 (2000).
2. Magnuson, J. J. *et al. Science* **289**, 1743–1746 (2000).
3. Bradley, N. L., Leopold, A. C., Ross, J. & Huffaker, W. *Proc. Natl Acad. Sci. USA* **96**, 9701–9704 (1999).
4. Dunn, P. O. & Winkler, D. W. *Proc. R. Soc. Lond. B* **266**, 2487–2490 (1999).
5. Both, C. & Visser, M. E. *Nature* **411**, 296–298 (2001).
6. Beebe, T. J. C. *Nature* **374**, 219–220 (1995).
7. Crick, H. Q. P., Dudley, C., Glue, D. E. & Thomson, D. L. *Nature* **388**, 526 (1997).
8. McCleery, R. H. & Perrins, C. M. *Nature* **391**, 30–31 (1998).
9. Sagarin, R. & Micheli, F. *Science* **294**, 811 (2001).
10. Steel, D. *Marking Time* (Wiley, New York, 2000).
11. Sparks, T. H. *Int. J. Biometeorol.* **42**, 134–138 (1999).
12. Schwartz, M. D. *Int. J. Biometeorol.* **42**, 113–118 (1999).

Supplementary information accompanies this communication on Nature's website (www.nature.com).

Competing financial interests: declared none.

Brood parasitism

Female ducks can double their reproduction

By engaging in extra-pair matings, the most successful males in some bird species can father twice as many young as are present in their own nest^{1,2}. Here we describe a female parallel in goldeneye ducks (*Bucephala clangula*), whereby some females can double their reproductive output by combining brood parasitism with normal nesting. This huge reproductive advantage should create strong selection for parasitic tactics.

We used protein fingerprinting by electrophoresis³ to trace the egg-laying patterns of individual females and determine the maternity of eggs in nests at Lake Mjörn in southwest Sweden^{3,4}. We marked incubating females individually with steel and colour rings, recorded the hatching success of clutches, marked chicks' wings with numbered tags and brood-specific coloured tape, and recorded their survival over four weeks.

Numerical results are shown in Fig. 1a and represent the most intensive study year, 1986, in which the success of the different female tactics⁵ could be accurately estimated in a core study area from albumen samples taken from all 383 goldeneye eggs in 36 nests (7 of which were not incubated).

Three different female tactics seemed to be equally common: non-parasitic nesting, pure parasitism (laying only in the nests of other females), and parasitism followed by nesting ('nesting parasites') (Fig. 1). Observations of unmarked broods indicated that about 10% of nests were not discovered, and that edge effects may have led to underestimation of nesting parasites.

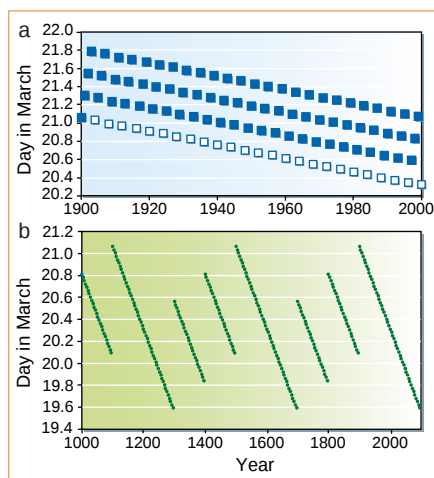


Figure 1 Trends in the calendar date of the vernal equinox (VE). Data are from a model produced by the Goddard Institute for Space Studies (<http://aom.giss.nasa.gov/svernal.html>). The model hindcasts and forecasts the times of equinoxes on the basis of a constant VE year of 365.2425 days. The actual VE year varies, and is currently closer to 365.2422 days; this discrepancy has a negligible effect on the bias reported here. **a**, Data for 1900–2000. The trend is driven by leap years (hollow symbols), which overcorrect for the yearly difference between the VE year and the 365-day calendar. **b**, Data for 1000–2099; one year out of every four is plotted. Because 2000 was a leap year, the trend towards earlier VE will continue until 2100.

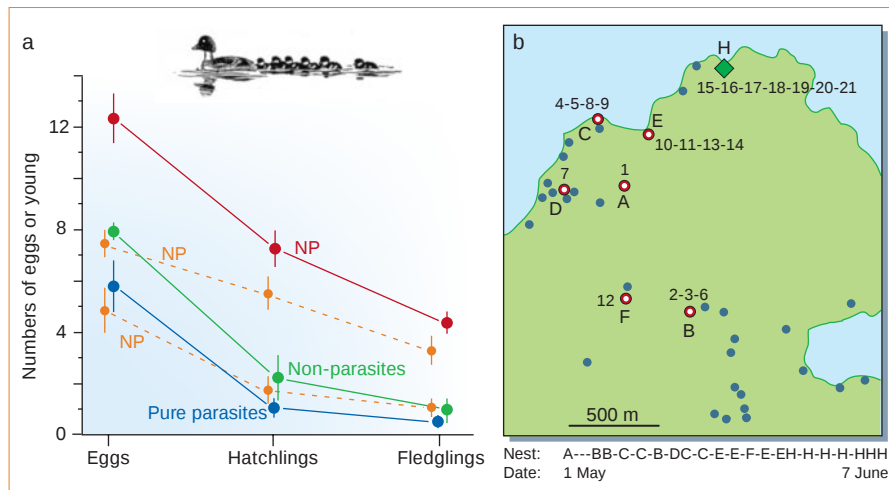


Figure 1 Reproductive tactics of female goldeneye ducks. **a**, Reproductive success (means $\pm$ s.e.). Nesting parasites (NP; $n=15$) first laid eggs in another female's nest (parasitic, lower dashed orange line), and then in their own nest (parental, higher dashed orange line), producing in total (red line) many more eggs, hatchlings and fledglings per female than did pure parasites (blue; $n=15$) and non-parasites (green; $n=14$). Differences among the categories of female are statistically significant at each stage (eggs, hatchlings and fledglings; $P<0.005$ after experiment-wise Dunn–Sidak correction). **b**, Distribution of parasitic laying sites (red, A–F) of the most successful nesting parasite in 1986, who laid 21 eggs in total. Numbers show the order in which she laid these eggs; grey dots represent other nest boxes. Date and nest site for each egg are shown at the bottom (dashes indicate days on which this female laid no eggs); the female laid her first parasitic egg (designated as 1 in nest A) on 1 May, the second (2) in another nest (B) on 5 May, and so on. After laying 14 parasitic eggs, the female laid and incubated 7 eggs (15–21) in her own nest (H, green square).

For 4 out of 15 nesting parasites, the shortest distance between a nest where the female had laid and the edge of the core study area was less than the median (for nesting parasites) of the maximum distance between a female's own nest and a nest that she parasitized.

The three tactics differed strongly in reproductive success (Fig. 1a). Nesting parasites laid 12.3 eggs, 1.5 times as many as non-parasites (7.9), and twice as many as pure parasites (5.8). The most successful female laid 21 eggs (Fig. 1b), almost twice the previously supposed maximum for the species. In terms of chicks leaving the nest, nesting parasites out-reproduced non-parasitic females and pure parasites by more than 3:1 and 6:1 (7.3, 2.2 and 1.1 chicks, respectively). There is evidence that parasitic chicks survive just as well as the host's own chicks⁶, in which case the advantage of nesting parasites is also present at the four-week stage (Fig. 1a).

How did nesting parasites achieve their high success? Their parasitic eggs resulted in fledgling numbers that are comparable to those from the host's eggs. Also, nesting parasites later laid their own clutch, which was more successful than the earlier clutches of non-parasitic females as the risk of parasitism-related disturbance and nest desertion or predation decreased over the season. Eggs hatched out in 13 of 15 nesting-parasite nests, but in only 5 of 14 non-parasite nests ($P=0.0078$).

There is no evidence that reduced survival or reproduction in later years nullifies the reproductive advantage of nesting parasitism, and there were no obviously

special circumstances in 1986 that would have promoted the success of nesting parasites. Their proportions were comparable in other years (our unpublished results).

These results point to a fitness advantage for nesting parasites: by combining parasitism with normal nesting, some female goldeneye ducks more than doubled their reproduction. This raises questions as to why females differ in their reproductive tactics and about the long-term fitness costs and benefits of the different tactics. There is evidence that tactics are flexible — we found that many females switched tactics between years (our unpublished results; see also refs 6–10). Nesting parasites may also be in their prime (with high egg-laying capacity). Nesting parasites had been breeding for fewer previous years (1.9 ± 0.29) than non-parasitic females (2.7 ± 0.60), but the difference is not statistically significant ($P=0.24$). The implications of the different reproductive strategies need further investigation.

Matti Åhlund, Malte Andersson

Department of Zoology, Gothenburg University,
Box 463, 405 30 Göteborg, Sweden
e-mail: malte.andersson@zool.gu.se

1. Kempaers, B. et al. *Nature* **357**, 494–496 (1992).
2. Sheldon, B. C. & Ellegren, H. *Anim. Behav.* **57**, 285–298 (1999).
3. Andersson, M. & Åhlund, M. *Ecology* **82**, 1433–1442 (2001).
4. Andersson, M. & Åhlund, M. *Proc. Natl Acad. Sci. USA* **97**, 13188–13193 (2000).
5. Eadie, J. M., Kehoe, F. P. & Nudds, T. N. *Can. J. Zool.* **66**, 1709–1721 (1988).
6. Eadie, J. M. Alternative reproductive tactics in a precocial bird: the ecology and evolution of brood parasitism in goldeneyes. Thesis, Univ. British Columbia (1989).
7. Sorenson, M. D. *Anim. Behav.* **42**, 771–796 (1991).
8. Lyon, B. C. *Anim. Behav.* **46**, 911–928 (1993).
9. Brown, C. R. & Brown, M. B. *Behav. Ecol.* **9**, 158–171 (1998).
10. McRae, S. B. *Behav. Ecol.* **9**, 93–100 (1998).

COMMUNICATIONS ARISING
Invertebrate evolution

Acaenoplax — polychaete or mollusc?

Palaeozoic invertebrate fossils may pose severe problems in assigning them to Recent taxa. Sutton *et al.*¹ describe the beautifully preserved and illustrated Silurian fossil *Acaenoplax hayae* as a “plated aplacophoran” mollusc, interpreting its polychaete-like characters as convergent features. In our opinion, it is more parsimonious to place this organism in the Polychaeta, as the molluscan similarities are limited to serial valve-like structures, suggesting polyplacophoran affinities. It is unlikely that *Acaenoplax* represents a primitive organization that is neither molluscan nor polychaete — instead, it appears to represent a highly derived, specialized line of invertebrate evolution.

There is no evidence that the seven dorsal valves and one ventral valve of *Acaenoplax* were mineralized and hence molluscan. If they were originally aragonitic and similar to, for example, the polyplacophoran *Mathevia*, they would at best represent highly specialized structures within polyplacophorans^{2–4}; the plates of Silurian polyplacophorans⁵ are quite different. In addition, spicules in aculiferan molluscs are aragonitic rather than cuticular¹ and are scale-shaped in all basal molluscan classes. Spiny spicules are developed only in advanced Solenogastres and Polyplacophora and never emerge from transverse ridges or from plates.

Caudofoveata are adapted to a freely burrowing lifestyle, are covered by tiled scales, and have lost all traces of seriality (pseudo-metamery of dorsoventral musculature). This contrasts with the also infaunal but tube-dwelling habit of many sedentary polychaetes. The posteriorly shifted pallial cavity in Caudofoveata houses a single pair of gills; only in Solenogastres do multiple ‘protuberances’ exist in a ventro-median (rather than terminal) pallial cavity. The terminal cavity of *Acaenoplax* is incompatible with the unrestricted, peripodal (surrounding the foot) pallial cavity of all known Polyplacophora. The combination of characteristics in *Acaenoplax*, when interpreted as a mollusc, results in a strange conglomerate that is not similar to Caudofoveata, to Polyplacophora, or to any primitive molluscan state¹.

The seriality common to Solenogastres and Polyplacophora refers only to their dorsoventral musculature, and affects neither the dorsal nor the lateroventral body region as in *Acaenoplax* (see Fig. 1j of ref. 1). Polyplacophora with reduced shell plates have mantle tissue that overgrows the plate margins; a ventral plate similar to that

in *Acaenoplax* is unknown, suggesting that *Acaenoplax* is a (semi-)sessile organism with an anchoring or opercular structure.

Solenogastres and Polyplacophora are ciliary gliders. In contrast, the ventral surface of *Acaenoplax* shows features of a muscularly creeping animal, which are typically found in contracted polychaetes. These features include transverse furrows and a shallow mid-ventral groove that marks the course of the ventral nervous system.

As many polychaete families have highly plastic morphology^{6,7}, the characteristics of *Acaenoplax* fit well within this frame. A ventral, cuticular shield that shows co-marginal accretion is present in the Sternaspidae⁷ (Fig. 1a); dorsal plates, referred to as elytra, formed by the parapodia and often bearing papillae, are diagnostic for the Aphroditidae⁷. For analogous dorsally fused parapodia, see *Chaetopterus variopedatus* (Chaetopteridae; Fig. 1b) and representatives of the Chrysopetalidae, Euphrosinidae and Spintheridae⁷. *Chaetozone* (Cirratulidae; Fig. 1c) has almost completely annular parapodial structures^{7,8}. These analogies may be no better than those with molluscs, but they are also no worse. Interpreting *Acaenoplax hayae* as a highly specialized polychaete thus avoids most of the uncertainties involved in assuming that it is a mollusc.

The polychaete characteristics of *Acaenoplax hayae* in its original description are: serially (segmentally) arranged parapodial appendages with chaetae; cuticle-covered, transversally wrinkled ventral surface with a longitudinal groove, here interpreted as a clear indication of a closed

dermo-muscular tube (*Hautmuskelschlauch*) and transversal musculature, with the midventrally positioned nervous system; dorsal, cuticular plates resembling elytra; modified parapodia adapted for mobility and anchoring in a tube (as in Flabelligeridae, for example); and a terminal 'opercular' structure (Sedentaria).

**Gerhard Steiner,
Luitfried Salvini-Plawen**

*Institute of Zoology, University of Vienna,
Althanstrasse 14, A-1090 Vienna, Austria
e-mail: gerhard.steiner@univie.ac.at*

1. Sutton, M. D., Briggs, D. E. G., Siveter, D. J. & Siveter, D. J. *Nature* **410**, 461–463 (2001).
2. Salvini-Plawen, L. *Malacologia* **19**, 249–278 (1980).
3. Salvini-Plawen, L. *Iberus* **9**, 1–33 (1990).
4. Runnegar, B. & Pojeta, J. Jr in *The Mollusca* Vol. 10 (eds Trueman, E. R. & Clarke, M. R.) 1–57 (Academic, Orlando, 1985).
5. Cherns, L. *Palaeontology* **41**, 939–974 (1998).
6. Fauchald, K. & Rouse, G. W. *Zool. Scripta* **26**, 71–138 (1997).
7. Rouse, G. W. & Fauchald, K. *Zool. Scripta* **26**, 139–204 (1997).
8. Hartmann-Schröder, G. in *Die Tierwelt Deutschlands* 58 2nd edn, 1–594 (Fischer, Jena, 1996).
9. Banse, K. & Riedl, R. in *Fauna und Flora des Mittelmeeres* (ed. Riedl, R.) 391–423 (Parey, Hamburg and Berlin, 1983).

Sutton et al. reply — Steiner and Salvini-Plawen claim that *Acaenoplax* cannot be a mollusc because it would sit uneasily within the caudofoveates, solenogastres or polyplacophorans; however, the fossil record frequently reveals character combinations that have not survived to Recent times¹. Although *Acaenoplax* displays a mosaic of molluscan characters that does not accord with any single modern group, this does not necessarily eliminate it from the phylum as a whole.

Aragonitic valves that grow by marginal accretion are highly characteristic of molluscs and were undoubtedly present in *Acaenoplax*. Valves of the closely related genera *Arctoplax*, *Heloplax* and *Enetoplax* occur in coeval deposits on Götland². These are preserved in a manner that is identical to that of co-occurring molluscan shells of original aragonitic composition (such as the valves of polyplacophorans)^{2,3} and display clear co-marginal growth lines. The composition of the spines of *Acaenoplax* is less certain, but they were rigid and sharp (see Fig. 2d in ref. 4) and hence were probably biomineralized, and those that arise from the cuticle are preserved in the same manner as those that project from valves. Our use of the term 'cuticular' was intended to refer to the occurrence of the spicules on the cuticle, rather than to their composition.

The groove on the ventral surface is the one feature of *Acaenoplax* that appears to be more polychaete-like than molluscan, although this morphology is typically associated with errant rather than sedentary polychaetes. The other proposed polychaete characters are problematic. Although there is clearly a serial structure in *Acaenoplax*,

there are no external signs of segmentation. Indeed, the overlap between subsequent 'lobe chevrons' is incompatible with true segmentation. The lobes and ridges of *Acaenoplax* are difficult to homologize with parapodial structures, as there are up to eight pairs of lobes per ridge. As spines arise from lobes, ridges and the cuticle (see Fig. 2d in ref. 4), they cannot be homologous with chaetae, which arise only from parapodia⁵.

Acaenoplax has multiple projections that emerge from a cavity at its larger termination. These features are very hard to homologize with polychaete posterior structures, so if *Acaenoplax* is a polychaete, then this termination must represent the anterior. Many sedentary polychaetes do have an anterior (prostomial) cavity from which feeding tentacles emerge⁵. There is, however, no evidence that the projections could extend significantly from the cavity and, with this anterior–posterior polarity, the spine array faces forwards, which is unlikely on functional grounds. By contrast, interpreting this termination as an aplacophoran-like mantle cavity containing gills is compatible with both the observed mobility of the projections and the direction of the spines.

Several *Acaenoplax* structures (such as lobes, ridges and the postero-ventral plate) lack convincing polychaete or molluscan homologues, and hence are considered to be autapomorphic and phylogenetically uninformative. Nonetheless, this leaves four molluscan characters (polyplacophoran-like aragonitic scleritome, spicules arising from cuticle and probably composed of aragonite, serial rather than segmented structure, and posterior cavity with projections) arrayed against one polychaete-like character (the ventral surface). We consider that the balance of evidence places *Acaenoplax* firmly within the Mollusca rather than in the Polychaeta.

Mark Sutton*, Derek E. G. Briggs†, David J. Siveter‡, Derek J. Siveter§

**Earth Sciences Department, University of Oxford, South Parks Road, Oxford OX1 3PS, UK.
e-mail: mark.sutton@earth.ox.ac.uk*

†Department of Earth Sciences, Wills Memorial Building, Queen's Road, Bristol BS8 1RJ, UK.

‡Department of Geology, Bennett Building, University of Leicester, University Road, Leicester LE1 7RH, UK.

§Geological Collections, University Museum of Natural History, Oxford OX1 3PW, UK.

1. Wills, M. A., Briggs, D. E. G., Fortey, R. A., Wilkinson, M. & Sneath, P. H. A. in *Fossils and Phylogeny* (ed. Edgecombe, G. E.) 33–105 (Columbia Univ. Press, New York, 1997).
2. Cherns, L. *Palaeontology* **41**, 939–974 (1998).
3. Cherns, L. *Palaeontology* **41**, 545–573 (1998).
4. Sutton, M. D., Briggs, D. E. G., Siveter, D. J. & Siveter, D. J. *Nature* **410**, 461–463 (2001).
5. Ruppert, E. E. & Barnes, R. D. in *Invertebrate Zoology* 6th edn, 508–554 (Saunders College, Orlando, Florida, 1994).

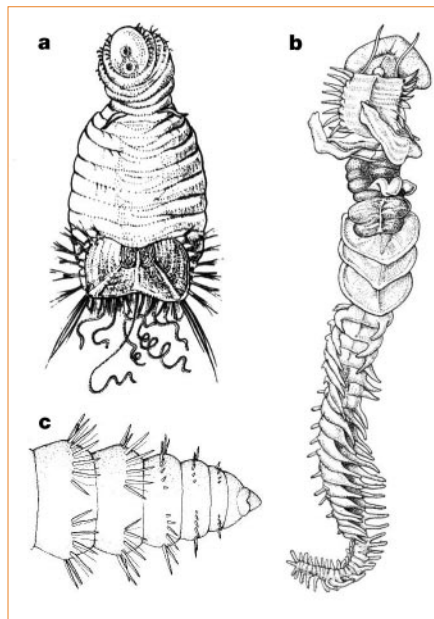


Figure 1 Polychaete examples of *Acaenoplax* characteristics. **a**, *Sternaspis scutata* (Sternaspidae), with ventro-posterior shield showing co-marginal accretion⁹. **b**, *Chaetopterus variopedatus* (Chaetopteridae), with dorsally fused parapodia⁹. **c**, *Chaetozone setosa* (Cirratulidae), with near-annular parapodia⁷.

Physical, chemical and biological processes in Lake Vostok and other Antarctic subglacial lakes

Martin J. Siebert*, J. Cynan Ellis-Evans†, Martyn Tranter*, Christoph Mayer‡, Jean-Robert Petit§, Andrey Salamatín|| & John C. Priscu¶

* Bristol Glaciology Centre, School of Geographical Sciences, University of Bristol, Bristol BS8 1SS, UK

† Freshwater Ecology Group, Biosciences Division, British Antarctic Survey, High Cross, Madingley Road, Cambridge CB3 0ET, UK

‡ Institute for Meteorology and Geophysics, University of Innsbruck, Innrain 52, A-6020 Innsbruck, Austria

§ Laboratoire de Glaciologie et Géophysique de l'Environnement, CNRS, BP96, 38402, Saint Martin d'Hères Cedex, France

|| Department of Applied Mathematics, Kazan State University, 18 Kremlyevskaya Str., Kazan 420008, Russia

¶ Land Resources and Environmental Sciences, 334 Leon Johnson Hall, Montana State University, Bozeman, Montana 59717, USA

Over 70 lakes have now been identified beneath the Antarctic ice sheet. Although water from none of the lakes has been sampled directly, analysis of lake ice frozen (accreted) to the underside of the ice sheet above Lake Vostok, the largest of these lakes, has allowed inferences to be made on lake water chemistry and has revealed small quantities of microbes. These findings suggest that Lake Vostok is an extreme, yet viable, environment for life. All subglacial lakes are subject to high pressure (~350 atmospheres), low temperatures (about -3°C) and permanent darkness. Any microbes present must therefore use chemical sources to power biological processes. Importantly, dissolved oxygen is available at least at the lake surface, from equilibration with air hydrates released from melting basal glacier ice. Microbes found in Lake Vostok's accreted ice are relatively modern, but the probability of ancient lake-floor sediments leads to a possibility of a very old biota at the base of subglacial lakes.

Only five years ago, Antarctic subglacial lakes were catalogued from airborne radio-echo sounding (RES) records collected in the 1970s (refs 1, 2) (Fig. 1). The largest of these, Lake Vostok, has generated a huge amount of scientific interest as a potential and unusual habitat for life. Quantification of the physical and chemical processes within Lake Vostok, and identification of life within the lake, requires samples of lake water. It was unclear in 1996 how, or even whether, water sampling could be performed. Recently, however, coring at Vostok station³, located at one end of Lake Vostok, has sampled ice down to 150 m from the surface of the lake. The lower 60 m of the ice core, and therefore the basal 210 m of the ice sheet beneath Vostok station, comprises ice refrozen from lake water⁴. This basal ice is, essentially, a sample of Lake Vostok.

Several independent analyses of the accreted ice have revealed preliminary information about the lake's chemistry and biology^{5–7}. However, these results, which need to be fully confirmed, have yet to be either integrated or interpreted with a full appreciation of the physical dynamics operating in Lake Vostok. It is therefore appropriate to review the recent research on Lake Vostok, in order to establish the main physical and chemical processes within the lake, and assess the implications of these for life in this extreme environment and in other subglacial lakes.

All subglacial lakes have an ice–water interface sloping at eleven times the ice surface gradient (but in the opposite direction), if they are in hydrostatic equilibrium. The pressure melting point along the upper boundary of the lake, which is dependent on the overlying ice thickness, therefore varies. For example, the pressure melting point in the south of Lake Vostok will be around 0.3°C less than that in the north. This leads to density contrasts between meltwater and the main lake-water body, and water circulation even within small lakes. Lake Vostok is the only subglacial lake that is known to have a substantial water depth (hundreds of metres)⁸. The maximum depths of other lakes are yet to be established but minimum water depths of at least seven lakes have been shown to be between 10 and

20 m (ref. 9). The bedrock slopes at the edges of subglacial lakes are often similar to those bordering Lake Vostok, and hence the water depths of many of the smaller lakes may be significantly greater than 20 m (ref. 10). We therefore contend that the physical and chemical processes that occur in Lake Vostok are likely to be applicable in many other subglacial lakes.

Lake age and physiography

Lake Vostok is at least 240 km long and 50 km wide, and lies between 3,750 m (over the south of the lake) and 4,150 m (over the north) beneath the central east Antarctic ice sheet (Fig. 2)^{11–13}. The lake is located within a very large subglacial topographic basin, similar to a rift valley, though it is unlikely that the rift valley tectonics remain active¹⁴. The basin has a crescent-like shape, and the side-walls are relatively steep (with gradients up to ~ 0.1) and high (up to $\sim 1,000$ m above the surface of the lake). Lake Vostok is at least 1,000 m deep in the south (Fig. 2), and relatively shallow in the north and extreme southwest. There may be several hundred metres of glacial sediments draped over its floor¹⁵. Although there are several similar subglacial basins in East Antarctica (for example, the Adventure subglacial trench and the Astrolabe subglacial basin), only Lake Vostok's basin is filled with deep water.

Lake Vostok is thought to have existed for as long as the ice sheet has been at a continental scale¹⁶. This is because, even over glacial–interglacial cycles, the ice thickness and subglacial conditions would not have changed significantly¹⁷. Some argue that the east Antarctic ice sheet has been stable for the last 20 million years at least¹⁸. If this is correct, Lake Vostok may have been in continual existence across the youngest third of the Cenozoic era and the entire Quaternary period. It is also possible that sediments across the floor of the valley may not have been completely scoured by the glacial advance of the infant Antarctic ice mass 33 million years ago¹⁶. This would mean that a historical record and biotic reservoir from the mid-Cenozoic is present at the base of Lake Vostok's sediment profile.

The age of the basal ice in the Vostok ice core is an important

constraint on the age of youngest water within the lake. Preliminary examination of the isotope record⁴, estimates of the air-hydrate crystal growth rates¹⁹ and ice flow modelling²⁰ provide evidence that the basal glacier ice could be as old as 1,000,000 years. This marks the maximum possible age of the youngest lake water. This also effectively marks the date at which Lake Vostok was last in direct contact with the biotic and chemical constituents of the Earth's atmosphere. The mean age of water within Lake Vostok is a function of the residence time of the water and how well the meltwater mixes with existing lake water. We speculate that if 20% of the annual meltwater mixes with the resident lake water before refreezing, then the residence time of Lake Vostok would be around 100,000 years (ref. 21). Hence the mean age of Lake Vostok's water is most probably of the order of 1 million years old.

Ice flows onto Lake Vostok from the ridge B ice divide, located approximately 200 km from the lake's western margin^{8,13,22}. Surface ice motion across Lake Vostok has been measured using repeat-pass synthetic aperture radar interferometry (InSAR) from the ERS-1 satellite²² (Fig. 2). At Vostok station, the surface ice velocity is measured at 4.2 m yr^{-1} . The distance along the ice flowline between the western shoreline of Lake Vostok and Vostok station is about 15 km (Fig. 3). Thus surface ice will take about 5,000 years to cross the lake to the station. However, it may take longer for basal ice to

make this journey because grounding lines and mid-lake 'islands' (Fig. 3) may inhibit flow.

Borehole temperature measurements along the full length of the Vostok ice core have been used to establish the energy balance between the ice sheet and the lake^{17,23}. The mean basal temperature gradient is about 0.02 °C m^{-1} , which relates to an outward heat flux through the ice from the lake ceiling of 46 mW m^{-2} , indicating that rates of subglacial freezing beneath the Vostok ice core site are probably around 4 mm yr^{-1} . In the extreme case that basal ice at -10 °C flows over the western lake margin, the rates of freezing beneath Vostok station will probably not be higher than about 11 mm yr^{-1} .

The spatial distribution of subglacial melting and freezing can theoretically be estimated from isochronous internal radar layering, by observing the loss or gain of basal ice along a flowline. Using this technique, it has been shown that subglacial melting occurs in the north of Lake Vostok and freezing takes place in the south¹¹.

Lake salinity and water circulation

The pattern of density-induced water circulation within Lake Vostok may be influenced significantly by lake salinity. It is highly likely that the salinity of Lake Vostok varies vertically and horizontally. In order to understand the circulation in Lake Vostok, we must

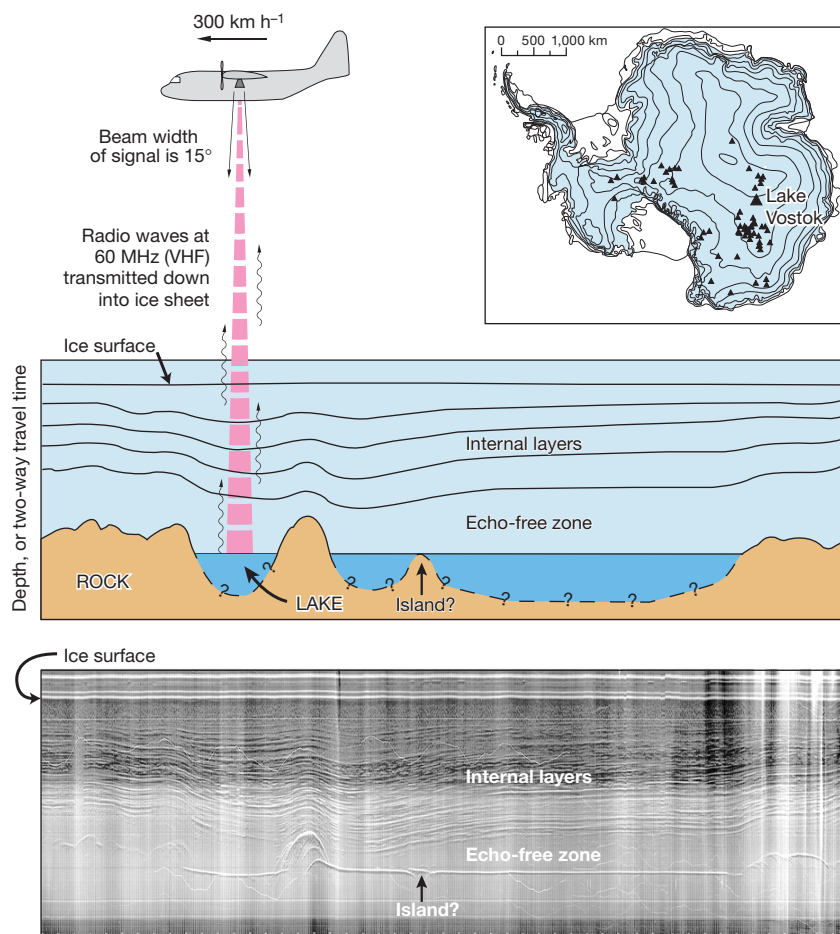


Figure 1 The technique of airborne radio-echo sounding, and its application to identifying Lake Vostok and other subglacial lakes. In the 1970s, airborne RES surveys were undertaken with a C130 Hercules transporter aircraft, with the wings mounted with the radar transmitter and receiver. Aircraft navigation was accurate to around 5 km in the centre of Antarctica. Today, most RES surveys use smaller aircraft and the

global positioning system (GPS) to navigate. Subglacial lakes are easily identified on airborne RES records owing to their uniformly strong and flat appearance². Bedrock perturbations are recorded as hyperbolae in radar data. The geographical location of known subglacial lakes (shown as triangles) is also provided¹.

evaluate how it would behave in the end-member scenarios of fresh and saline conditions. Evidence in support of saline water comes from the accreted ice from which an upper limit salinity of 0.4–1.2‰ has been estimated⁷. However, not all studies of the accreted ice agree with this, and some have argued that the accreted ice suggests that the lake waters are very pure⁶. Very high frequency (VHF) radio-wave penetration has been observed through up to 20 m of lake water in the north of Lake Vostok (where melting occurs), which could only be possible if the lake water is extremely pure⁹.

The circulation of pure water in Lake Vostok will be driven by the differences between the density of meltwater and lake water. Geothermal heating will warm the bottom water to a temperature higher than that of the upper layers. The water density will decrease with increasing temperature, resulting in an unstable water column²⁴. This leads to vertical convective circulation in the lake, in which cold meltwater sinks down the water column and water warmed by geothermal heat ascends up the water column (Fig. 4a). However, in the south, where the ice sheet is thinner and subglacial freezing takes place, a pool of slightly warmer and stratified water may occur below the ice roof²⁴. Here, the water would not be involved in the convective motion as heat is transferred from the ice towards the lake (that is, the temperature will decrease with depth).

There have been three numerical models from which the circulation of pure water in Lake Vostok can be evaluated^{24–26} (Fig. 4a). The models indicate that in the northern area of Lake Vostok, where the ice is thickest, meltwater will be colder and denser than both the surrounding lake water and meltwater in areas with thinner ice cover. It appears therefore that this region is the main zone of downwelling of pure water. The models agree that northern meltwater will sink and be transported horizontally to the south, via a

clockwise circulation system, to a region where the pressure-melting point is higher (Fig. 4a).

In the case where the lake is saline to some extent⁷, the fresh meltwater will be buoyant compared with the resident lake water (Fig. 4b). Because of this, the northern meltwater is likely to spread southwards and upwards travelling into regions of progressively lower pressure, displacing lake water in the south if the horizontal salinity gradient (north–south) is high enough to compensate for geothermal warming. The possibility of such a regime is controlled by (1) the melting–freezing rates, (2) the rates of mixing between the fresh ascending meltwater layer and the underlying saline water, and (3) the vertical free convection driven by the geothermal heating of water at the lake bottom. If the heat flux from the basal water is not sufficiently high, the cold northern water will eventually enter a region where its temperature is at or below the pressure melting point. From this point on, the water will refreeze back onto the ice-sheet underside, some distance away from where it was first melted into the lake. In this case, a conveyor of fresh cool meltwater from north to south directly beneath the ice ceiling of Lake Vostok will be set up, which causes displacement of warmer dense lake water from south to north. Otherwise, if the bulk salinity is not high enough, a stable stratification will develop in the upper water layers below the tilted lake ceiling, with more saline warmer water in the south and fresher, cooler water in the north²⁴. The deep-water stratum will be subject to vertical thermal-haline convection because, for any reasonable level of its salinity, the temperature at the lake bottom will be high enough to initiate convection.

Regardless of whether Lake Vostok is saline or fresh, meltwater from the north will be transferred towards the freezing zone in the south, and water will flow from the surface into the main body of the

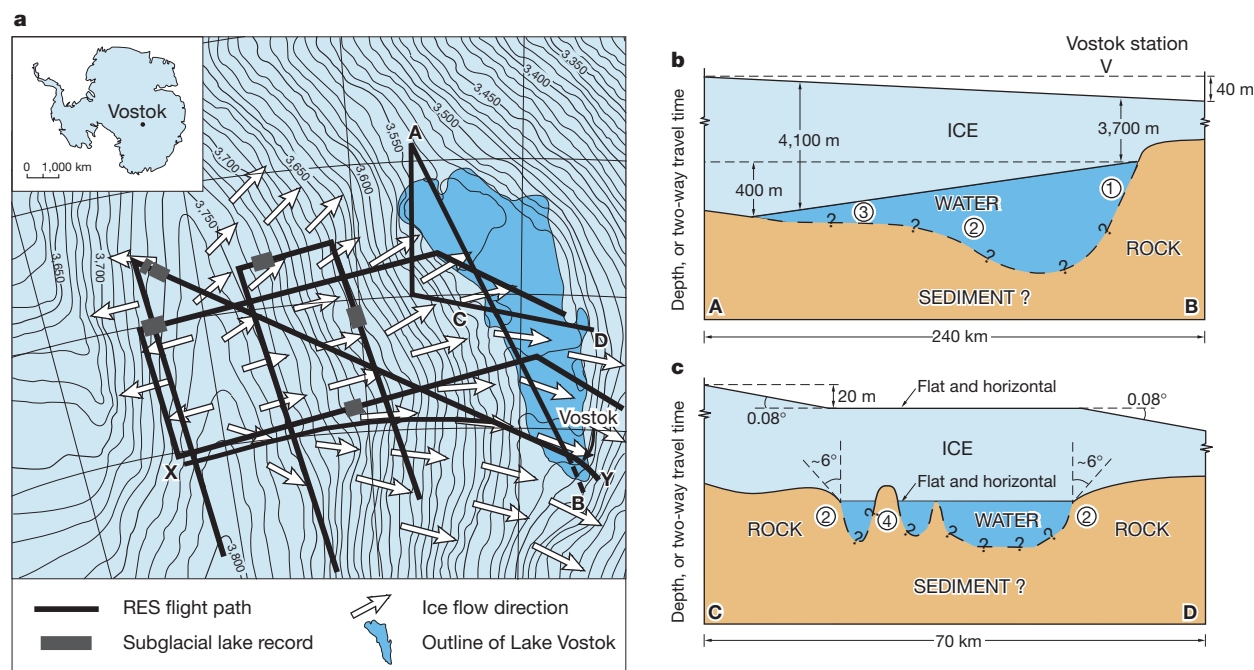


Figure 2 The dimensions and topographic setting of Lake Vostok. **a**, ERS-1 altimetry of the Antarctic ice sheet between ridge B and dome C. The location of Lake Vostok can be identified from the anomalous flat ice-surface region. The contour interval is 10 m. SPRI (Scott Polar Research Institute) RES flight lines and the location of all known subglacial lakes around Lake Vostok (shown as black squares) are provided. Arrows denote the direction of surface flow of ice over Lake Vostok calculated from InSAR^{11,12}. **b**, Cross-section from north to south along the 200 km length of the lake.

c, Cross-section from west to east along the 50 km width of the lake. The depth of Lake Vostok can be estimated by: (1) seismic information¹⁵, which has revealed a water depth of over 500 m beneath Vostok station and ~1000 m to the north of the station; (2) a side-wall bedrock gradient adjacent to the lake of 0.1, which indicates several hundred metres of water depth in the centre of the lake; (3) radiowave reflections from the lake floor, showing the water depth to be between 10 and 20 m in the north of the lake; and (4) bedrock 'islands' measured by RES.

lake before it returns to the north. This process is critical to evaluating the chemical and biological information stored within the accreted ice beneath Vostok station.

Lake water chemistry and life-supporting nutrients

Solutes are added to the water in Lake Vostok during ice melt and via chemical weathering of debris in and around the base of the lake.

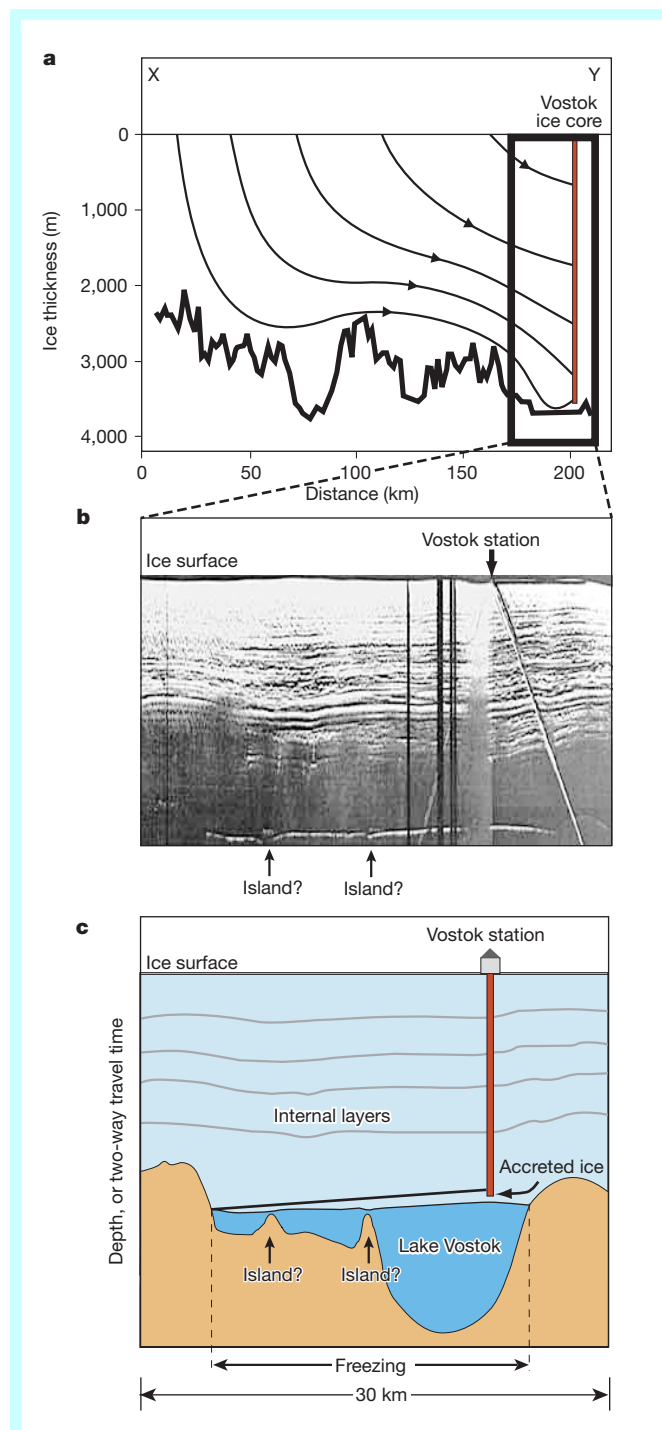


Figure 3 Ice sheet cross-sections along the line of ice flow from the ice divide, across Lake Vostok, to the Vostok ice core. **a**, Suggested ice particle flowpaths between ridge B and the southern end of Lake Vostok (Fig. 1a). The bedrock elevation has been measured from airborne RES². **b**, Raw records along an ice surface flowline across Lake Vostok, showing the pattern of internal layering. Also shown are disturbances to the otherwise smooth ice-sheet base above the lake, which may reflect shallow lake conditions and ice-sheet grounding. **c**, Interpretation of the RES data provided in **b**.

Solute is rejected from the ice lattice during freezing²⁷, and hence there should be an accumulation of solute in the lake water over time. The isotopic and major ion composition of Lake Vostok can be inferred from the composition of the accreted ice, provided assumptions are made about the percentage of lake water entombed within growing ice crystals and the partitioning of solute between lake water and ice^{6,7}. Geochemical investigations of the accreted ice indicate that near-surface water at the southern end of Lake Vostok has a composition comparable with water sampled directly from subglacial environments^{6,28} (Na^+ , 200–700 microequivalents per litre ($\mu\text{eq l}^{-1}$); Ca^{2+} , 115–270 $\mu\text{eq l}^{-1}$; Mg^{2+} , 275–350 $\mu\text{eq l}^{-1}$; Cl^- , 54–461 $\mu\text{eq l}^{-1}$; SO_4^{2-} , 444–1,150 $\mu\text{eq l}^{-1}$; HCO_3^- , 300 $\mu\text{eq l}^{-1}$). This suggests that the principal reactions that supply solute to subglacial waters include silicate and carbonate hydrolysis, carbonation, sulphide oxidation and oxidation of organic carbon.

Gas hydrates (or clathrates; crystal lattices formed by water molecules around gas molecules under conditions of low temperatures and high pressures²⁹) have the potential to store significant quantities of gas in Lake Vostok. Hydrates are known to be present in the glacial ice above the lake^{30,31} and have also been observed in the accreted ice (Fig. 5). Thus, gas hydrates should be present in Lake Vostok. If, as seems probable, the rate of hydrate supply to the lake through melting of glacial ice exceeds their loss through freezing within accreted ice, they will accumulate in the lake over time. Experimental studies of a range of gas hydrates suggest they may be stable within an environment such as Lake Vostok^{32,33}, in which the pressure is about 350 atm and the temperature is about -3°C . Some hydrates may have a lower density than the surrounding melt water and could therefore migrate beneath the sloping ice–water interface and accumulate in certain regions. Dissolved oxygen is expected in Lake Vostok's water owing to continuous equilibration with gas hydrates³³, making certain areas of the lake more oxic/suboxic than anoxic. This oxygenation will probably occur near the surface of the lake, proximal to the supply of hydrates from the melting ice sheet base. Water circulation (Fig. 4) will then allow the transfer of oxygenated water to other parts of the lake, including the southern side, where subglacial freezing occurs, and deeper regions.

Recent investigations into microbially mediated chemical weathering in subglacial environments have shown that such places, out of free contact with the atmosphere, can become progressively anoxic over time^{28,34}. This is because chemical oxidation and microbial respiration of organic matter deplete the original dissolved oxygen. The anion stoichiometry of the accreted ice is consistent with sulphide oxidation and carbonate dissolution being important chemical reactions in Lake Vostok. Further, the presence of dissolved organic carbon (DOC) in the accreted ice and therefore the lake water⁶ implies that biological oxidation of organic matter may occur within the lake, consuming the dissolved oxygen. This suggests that the concentration of dissolved oxygen will decrease with distance from the hydrate supply as the water flows around the lake. Bottom waters may therefore be suboxic compared with the surface and sediments would probably be anoxic. When the water eventually returns to the surface of the lake in the north, the dissolved oxygen would be replenished from the gas hydrate supplied by the melting ice sheet.

Thus, subglacial melting and refreezing above Lake Vostok leaves the water enriched with modest quantities of solute, maintains a reservoir of gas in hydrate form from which dissolved oxygen (and other gases) may be transferred to the water, and supplies sediment that will accumulate at the floor of the lake¹¹.

Life in Lake Vostok and other subglacial lakes

Solar radiation through photosynthesis is, directly or indirectly, the major source of energy for most organisms on Earth and a major driver of processes in surface Antarctic lake ecosystems^{35,36}. In its absence, microorganisms in subglacial lakes must use chemical

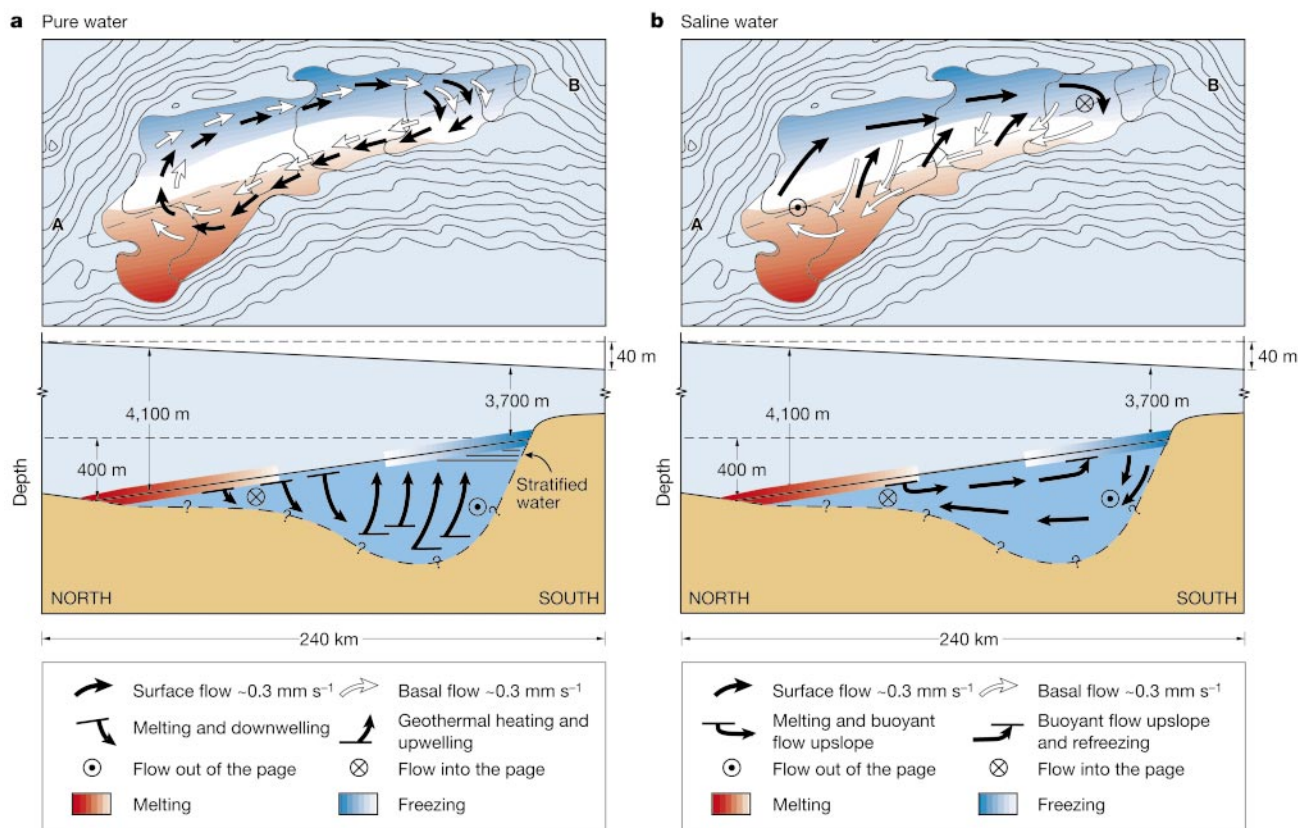


Figure 4 Water circulation patterns within Lake Vostok under fresh and saline conditions. **a**, Circulation calculated by numerical modelling, assuming that the water is pure^{24–26}. The white arrows show the bottom water circulation and the black arrows denote the higher level circulation close to the ice base. There are two clockwise circulation paths in the upper and lower regions of the lake. Most of the vertical mixing

takes place in the southern two-thirds of the cavity, but this exchange is rather limited. Blue shading refers to predicted zones of subglacial freezing; red shading indicates subglacial melting. **b**, Circulation of Lake Vostok, thought to occur as a result of saline conditions⁷ (that is, 1.2–0.4‰).

energy to power biological processes³⁷. To this end, the dissolved oxygen supplied from gas hydrates is particularly important, as it will be utilized by microbes via oxidation of organic matter and sulphides as a source of energy.

The Vostok accreted ice offered the first opportunity for aquatic biologists to investigate material derived from a subglacial lake. Several studies have shown that the accreted ice contains bacteria (Fig. 6)^{5,6} and low concentrations of inorganic and organic 'growth nutrients'⁵. Microbes released from melted ice cores of accretion ice are viable and have been shown to incorporate and respire ¹⁴C-labelled organic substrates⁵. The accreted ice originates from Lake Vostok, so these microbes, if they are not contaminants, must have been present in the lake water at some point, and survived there before being incorporated into accreted ice³⁸.

Molecular profiling of accreted ice microbes using 16s rDNA techniques^{6,38} show a very close agreement with present-day surface microbiota. This is consistent with the idea that the microbes found in accreted ice originate from deep glacial ice³⁹, are transported towards the south of the lake after melting out at the north end, and consequently spend little time in the lake before refreezing. These biota may be unrepresentative of lake microbes that are more likely to originate from lake-floor sediments and rocks. In contrast to the accreted ice microbiota, if lake-floor biota exist, their period of isolation may be sufficient for significant evolutionary divergence to have occurred⁴⁰, particularly given the potential selection pressures that exist within the subglacial environment.

Knowledge of the physical and chemical environment of the lake

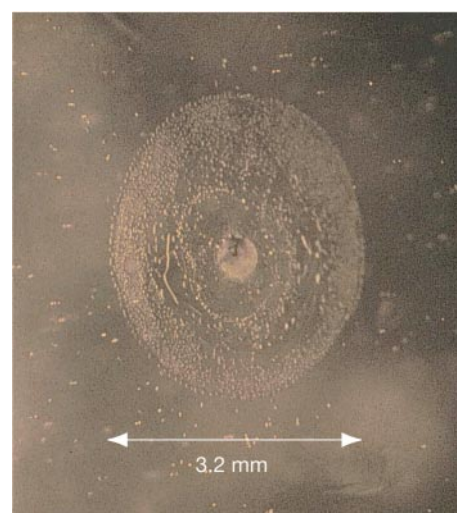


Figure 5 A microscope image of a gas hydrate (or clathrate) structure found in Lake Vostok's accreted ice 3,566 m below the ice-sheet surface, which is 174 m above the lake surface. Fifteen similar clathrate structures, between 3 and 5 mm in diameter, were identified within a 32-cm-long vertical section of the accreted ice. This structure is formed as the ice relaxes under normal atmospheric pressure, causing the hydrate to convert to gas and expand.

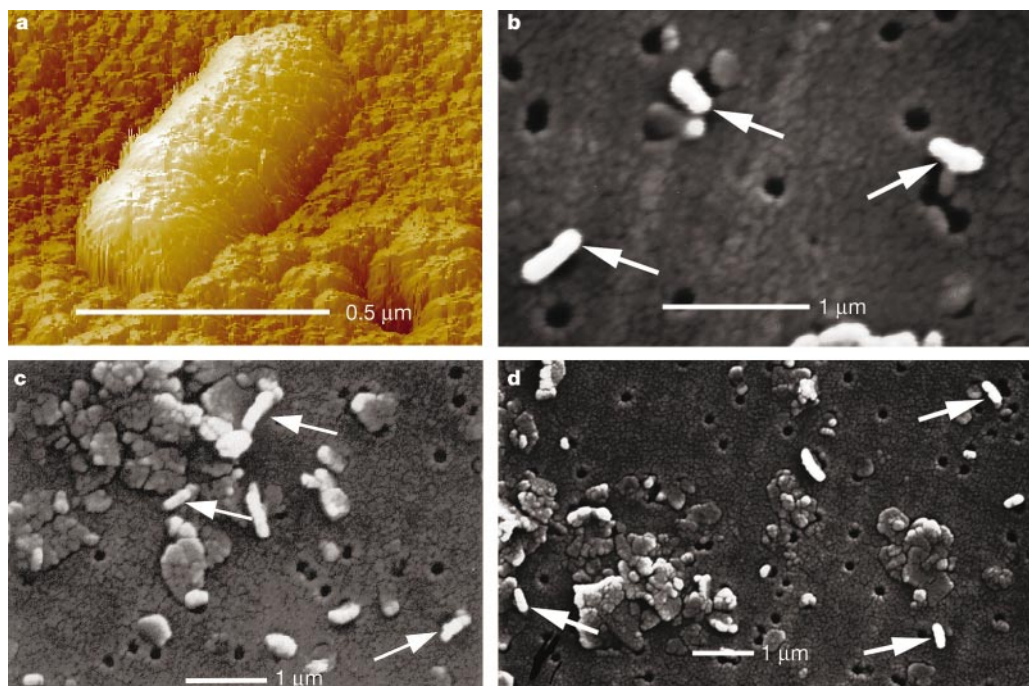


Figure 6 Images of bacteria frozen into Lake Vostok's accreted ice⁶. **a**, Atomic force microscope image of a single bacteria, illustrating how bacteria can be identified using this device. **b–d**, Scanning electron microscope images showing the occurrence of

rod-shaped bacteria (noted by arrows) within the accreted ice. The ice was sampled from the Vostok ice core at a depth of 3,590 m, which is about 151 m above the lake surface and 60 m below the glacial accreted ice boundary.

provides an insight to community structure. For example, barotolerance is commonly encountered in microorganisms, so the pressure environment of Lake Vostok does not necessarily represent a significant obstacle. However, the lake's pressure environment is well below that associated with obligate barophiles⁴¹, so this specialist group may not be present. The chemical composition of the lake water may provide adequate nutrients to support a heterotrophic microbial assemblage. DOC levels in accretion ice between 79 and 510 $\mu\text{g l}^{-1}$ (refs 5, 6) suggest that Lake Vostok itself may have a DOC level of up to 1,200 $\mu\text{g l}^{-1}$, which is adequate to support heterotrophic growth⁶. Inorganic nitrogen (nitrate plus nitrite) and total nitrogen levels ranging from 0.16 to 0.17 and 0.97 to 2.58 μM , respectively, have also been reported⁵, which could support active biogeochemical cycling of essential nutrients within the lake.

The suite of biogeochemical reactions occurring in the water column and sediments are dependent on the supply of oxidants and reductants (such as oxygen, nitrate, organic carbon, sulphides and ammonium). These are ultimately provided only via melting of the glacial ice sheet and weathering of sediments, and their rates of supply will be critical for governing the rates of biological activity. It has been postulated that extreme oxidant depletion occurred on the 'snowball earth', where aquatic environments were secluded from solar radiation and the supply of oxidants by the persistence of a global ice sheet for millions of years⁴². This would eventually lead to the virtual annihilation of ecosystems on timescales of tens of millions of years. The possibility that Lake Vostok could have existed for a comparable timescale since the onset of Antarctic glaciation (~33 million years ago) has prompted comparison with the snowball earth⁴³. However, the presence of microbes, dissolved oxygen and oxidants such as nitrate and sulphate, as well as the apparent utilization of nitrate in modern-day Lake Vostok⁶, suggest that a state of chemical disequilibrium can persist for timescales of

tens of millions of years, and that a range of biogeochemical processes based on chemical energy can likewise persist on these timescales.

The subglacial hydrology of the Antarctic ice sheet is not well known. However, the presence of subglacial lakes shows that large regions of the ice base are subject to melting⁴⁴. It is possible that neighbouring subglacial lakes could have a hydrological connection, feeding water from one lake to another. By contrast, groups of lakes that are distant from each other (such as those in dome C compared with ridge B (ref. 1)) will be completely isolated. If the biota of subglacial lakes originate from the ice above, then it is probable that all lakes contain a similar biota that could be subject to adaptation dependent on the specific conditions in each lake. However, if there are extant biota from preglacial times in the basal sediments, there is potential for evolutionary processes to yield assemblages unique to groups of lakes.

Further research

Our understanding of subglacial lakes has improved through the identification of key physical and chemical processes in Lake Vostok. Subglacial melting occurs in the north and freezing occurs in the south, resulting in about 210 m of ice accreted to the ice-sheet base at Vostok station. The slope of the lake roof is much greater than the ice surface, causing spatial variation in the temperature and density of water near the ceiling of the lake, which drives water from north to south. Microbes found within the accreted ice have DNA profiles similar to those of contemporary microbes, suggesting that these originate from the relatively young (<1 Myr ago) glacial ice and were transported upslope to the freezing zone after melting out. These microbes may be unrepresentative of the biota deeper in the lake, or within the lake-floor sediments, which could be much older. The chemistry of the lake water has been inferred from analysis of the accreted

ice. There is evidence that Lake Vostok's water contains dissolved oxygen, fed by a reservoir of oxygen in hydrate form from the ice above³³. Dissolved oxygen allows the oxidation of sulphides and organic matter. As the lake biota must survive in an environment of permanent darkness, high pressure (350 atm) and low temperature (-3°C), these reactions will, in the probable absence of hydrothermal activity¹⁴, provide their only source of energy. Significant DOC and nitrogen have been found in the accreted ice, which is adequate to support heterotrophic growth, and a microbial assemblage within the lake itself. It is therefore anticipated that subglacial lakes house a variety of microorganisms potentially unique to subglacial Antarctica and, if they are isolated hydrologically, unique to each lake.

Future research on subglacial lakes will focus on three themes. The first concerns definition of Lake Vostok's physical environment and, to this end, airborne RES surveys have been performed to better define the lake physiography^{45,46}, and seismic experiments have taken place to measure Lake Vostok's depth and basal sediments¹⁵. RES surveys of lakes in ridge B and dome C are also planned for the next few years. The second theme involves the further analysis of accreted ice, which is taking place at a number of laboratories, and will result in a better understanding of the type and origins of microbes within the lake, and the water and gas chemistry of Lake Vostok. Comprehension of subglacial lake systems will also benefit from numerical modelling of water circulation and chemical processes, and this is under way at several institutes. The third theme relates to the exploration of subglacial lakes, and the sampling of water and sediments. Such activity appears to be some time away as there are technological hurdles and environmental concerns that need to be addressed. However, prototype machinery is being developed in several countries. Subglacial lake research is a new area of study and, to oversee its development and to ensure the environmental issues are upheld, the Scientific Committee on Antarctic Research has commissioned recommendations for future activities⁴⁷ and arranged for a group of specialists to develop science and implementation plans (scheduled to meet in November 2001). The investigation of subglacial lakes requires significant multinational cooperation and interdisciplinarity. The preliminary investigations above have helped to define the next generation of research objectives, and it is likely that several exciting bio-geochemical-physical systems will be documented during the next decade. □

1. Siegert, M. J., Dowdeswell, J. A., Gorman, M. R. & McIntyre, N. F. An inventory of Antarctic subglacial lakes. *Ant. Science* **8**, 281–286 (1996).
2. Oswald, G. K. A. & Robin, G. de Q. Lakes beneath the Antarctic Ice Sheet. *Nature* **245**, 251–254 (1973).
3. Petit, J. R. *et al.* Climate atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature* **399**, 429–436 (1999).
4. Jouzel, J. *et al.* More than 200 meters of lake ice above subglacial Lake Vostok, Antarctica. *Science* **286**, 2138–2141 (1999).
5. Karl, D. M. *et al.* Microorganisms in the accreted ice of Lake Vostok, Antarctica. *Science* **286**, 2144–2147 (1999).
6. Priscu, J. C. *et al.* Geomicrobiology of subglacial ice above Lake Vostok, Antarctica. *Science* **286**, 2141–2144 (1999).
7. Souchez, R., Petit, J. R., Tison, J.-L., Jouzel, J. & Verbeke, V. Ice formation in subglacial Lake Vostok, Central Antarctica. *Earth Planet. Sci. Lett.* **181**, 529–538 (2000).
8. Kapitsa, A., Ridley, J. K., Robin, G. de Q., Siegert, M. J. & Zotikov, I. A large deep freshwater lake beneath the ice of central East Antarctica. *Nature* **381**, 684–686 (1996).
9. Gorman, M. R. & Siegert, M. J. Penetration of Antarctic subglacial water masses by VHF electromagnetic pulses: estimates of minimum water depth and conductivity. *J. Geophys. Res.* **104**, 29311–29320 (1999).
10. Dowdeswell, J. A. & Siegert, M. J. The dimensions and topographic setting of Antarctic subglacial lakes and implications for large-scale water storage beneath continental ice sheets. *Geol. Soc. Am. Bull.* **111**, 254–263 (1999).
11. Siegert, M. J., Kwok, R., Mayer, C. & Hubbard, B. Water exchange between the subglacial Lake Vostok and the overlying ice sheet. *Nature* **403**, 643–646 (2000).
12. Siegert, M. J. & Kwok, R. Ice-sheet radar layering and the development of preferred crystal orientation fabrics between Lake Vostok and Ridge B, central East Antarctica. *Earth Planet. Sci. Lett.* **179**, 227–235 (2000).
13. Siegert, M. J. & Ridley, J. K. An analysis of the ice-sheet surface and subsurface topography above the Vostok Station subglacial lake, central East Antarctica. *J. Geophys. Res.* **103**, 10195–10208 (1998).
14. Jean-Baptiste, P., Petit, J. R., Lipenkov, V. Ya., Raynaud, D. & Barkov, N. I. Helium isotope in deep

Vostok ice core (Antarctica): constraints on hydrothermal processes and water exchange in the subglacial lake. *Nature* **411**, 460–462 (2001).

15. Lukin, V. V. *et al.* Rezultaty geofizicheskikh issledovaniy podlednikovogo ozera Vostok (Antarktida) v 1995–1999 gg. [Results of geophysical studies of subglacial Lake Vostok (Antarctica) in 1995–1999]. *Problemy Arktiki i Antarkтики* **72** (jubilee issue), 237–248 (2000) (in Russian).
16. Barrett, P. J. Antarctic palaeoenvironments through Cenozoic times. *Terra Antarctica* **3**, 103–119 (1996).
17. Salamatin, A. N. in *Physics of Ice Core Records* (ed. Hondoh, T.) 243–282 (Hokkaido Univ. Press, Sapporo, Japan, 2000).
18. Stroeve, A. P., Burckle, L. H., Kleman, J. & Prentice, M. L. Atmospheric transport of diatoms in the Antarctic Sirius Group: Pliocene deep freeze. *GSA Today* **8**, 1–5 (1998).
19. Lipenkov, V. Ya., Barkov, N. I. & Salamatin, A. N. Isotopiya klimata i oledneniya Antarktidy po rezul'tatam izucheniya ledanogo kerna so stantsii Vostok [The history of climate and glaciation of Antarctica from results of the ice core study at Vostok Station]. *Problemy Arktiki i Antarkтики* **72** (jubilee issue), 197–236 (2000) (in Russian).
20. Barkov, N. I., Vostrikov, R. N., Lipenkov, V. Ya. & Salamatin, A. N. Kolebaniya temperatury vozdukh i osadkov v rayone stantsii Vostok na protyazhenii chetyrykh klimaticheskikh tsykhlov za posledniye 420 tys. let [Air temperature and precipitation variation sin Vostok Station area through four climatic cycles during recent 420 kears]. *Arktika i Antarktika* **1** (in the press, 2001) (in Russian).
21. Mayer, C. & Siegert, M. J. Numerical modelling of ice-sheet dynamics across the Vostok subglacial lake, central East Antarctica. *J. Glaciol.* **46**, 197–205 (2000).
22. Kwok, R., Siegert, M. J. & Carsey, F. Ice motion over Lake Vostok. *J. Glaciol.* **46**, 689–694 (2000).
23. Salamatin, A. N., Vorstrikov, R. N., Petit, J. R., Lipenkov, V. Ya. & Barkov, N. I. Geophysical and paleoclimatic implications of the stacked temperature profile from the deep borehole at Vostok station, Antarctica. *Mater. Glyatsiol. Issled.* **85**, 233–240 (1998).
24. Wüest, A. & Carmack, E. A priori estimates of mixing and circulation in the hard-to-reach water body of Lake Vostok. *Ocean Model.* **2**, 29–49 (2000).
25. Williams, M. J. M. Application of a three-dimensional numerical model to Lake Vostok: An Antarctic subglacial lake. *Geophys. Res. Lett.* **28**, 531–534 (2001).
26. Mayer, C., Grosfeld, K. & Siegert, M. J. Water circulation and mass exchange within subglacial Lake Vostok. *Earth Planet. Sci. Lett.* (submitted).
27. Killawee, J. A., Fairchild, I. J., Tison, J.-L., Janssens, L. & Lorrain, R. Segregation of solutes and gases in experimental freezing of dilute solutions: implications for natural glacial systems. *Geochim. Cosmochim. Acta* **62**, 3637–3655 (1998).
28. Tranter, M. *et al.* Geochemical weathering at the bed of Haut Glacier d'Arolla, Switzerland—a new model. *Hydrol. Process.* (in the press).
29. Lee, S. Y. & Holder, G. D. A generalized model for calculating equilibrium states of gas hydrates. *Ann. NY Acad. Sci. USA* **912**, 614–622 (2000).
30. Uchida, T., Hondoh, T., Mae, S., Lipenkov, V. Ya. & Duval, P. Air hydrate crystals in deep ice-core samples from Vostok Station, Antarctica. *J. Glaciol.* **40**, 79–86 (1994).
31. Lipenkov, V. Ya. in *Physics of Ice Core Records* (ed. Hondoh, T.) 327–358 (Hokkaido Univ. Press, Sapporo, Japan, 2000).
32. Kuhs, W. F., Klapproth, A. & Chazallon, B. in *Physics of Ice Core Records* (ed. Hondoh, T.) 373–392 (Hokkaido Univ. Press, Sapporo, Japan, 2000).
33. Lipenkov, V. Ya. & Istomin, V. A. On the stability of air clathrate-hydrate crystals in subglacial Lake Vostok, Antarctica. *Mater. Glyatsiol. Issled.* **91** (in the press, 2001).
34. Bottrell, S. H. & Tranter, M. Sulphide oxidation under partially anoxic conditions at the bed of Haut Glacier d'Arolla, Switzerland. *Hydrol. Process.* (in the press).
35. Ellis-Evans, J. C. Microbial diversity and function in Antarctic freshwater ecosystems. *Biodiversity Conserv.* **5**, 1395–1431 (1996).
36. Priscu, J. C. *et al.* Carbon transformations in the water column of a perennially ice-covered Antarctic Lake. *Bioscience* **49**, 997–1008 (1999).
37. Rochschild, L. J. & Mancinelli, R. L. Life in extreme environments. *Nature* **409**, 1092–1101 (2001).
38. Christner, B. C., Mosley-Thompson, E., Thompson, L. G. & Reeve, J. V. Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice. *Appl. Env. Microbiol.* (in the press).
39. Abyzov, S. S., Mitskevich, I. N. & Poglazova, M. N. Microflora of the deep glacier horizons of central Antarctica. *Microbiology* **67**, 66–73 (1998).
40. Lawrence, J. G. & Ochman, H. Molecular archaeology of the *Escherichia coli* genome. *Proc. Natl. Acad. Sci. USA* **95**, 9413–9417 (1998).
41. Fang, J. S., Barcelona, M. J., Nogi, Y. & Kato, C. Biochemical implications and geochemical significance of novel phospholipids of the extremely barophilic bacteria from the Marianas Trench at 11,000 m. *Deep Sea Res.* **47**, 1173–1182 (2000).
42. Gaidos, E. J., Neelson, K. H. & Kirschvink, J. L. Life in ice-covered oceans. *Science* **284**, 1631–1633 (1999).
43. Hoffman, P. F., Kaufman, A. J., Halverson, G. P. & Schrag, D. P. A neo-proterozoic snowball earth. *Science* **281**, 1342–1346 (1998).
44. Siegert, M. J. Antarctic subglacial lakes. *Earth Sci. Rev.* **50**, 29–50 (2000).
45. Bell, R. E., Studinger, M., Tikku, A. A., Clarke, G. K. C. & Gutner, M. M. Evidence for open-system water exchange in subglacial Lake Vostok. *Nature* (submitted).
46. Tabacco, I. E. *et al.* Airborne radar survey above Lake Vostok region, central East Antarctica. Lake Geometry and internal layer analysis. *J. Glaciol.* (submitted).
47. Kennicutt, M. C. (ed.) Subglacial lake exploration: workshop report and recommendations. (Scientific Committee on Antarctic Research, 2001).

Acknowledgements

Many of the ideas developed in this review were formed during discussions at the International Conference on Antarctic Subglacial Lakes (1999), and two other international workshops: "Lake Vostok Study: Scientific Objectives and Technological Requirements" (1998); and "Lake Vostok: a curiosity or a focus for interdisciplinary study?" (1998). We thank the sponsors and organizers of these meetings (see <http://salegos-scar.montana.edu>).

Correspondence and requests for materials should be addressed to M.J.S. (e-mail: m.j.siegert@bristol.ac.uk).

Conserved signals and machinery for RNA transport in *Drosophila* oogenesis and embryogenesis

Simon L. Bullock & David Ish-Horowicz

Developmental Genetics Laboratory, Imperial Cancer Research Fund, PO Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

Localization of cytoplasmic messenger RNA transcripts is widely used to target proteins within cells. For many transcripts, localization depends on *cis*-acting elements within the transcripts and on microtubule-based motors; however, little is known about other components of the transport machinery or how these components recognize specific RNA cargoes. Here, we show that in *Drosophila* the same machinery and RNA signals drive specific accumulation of maternal RNAs in the early oocyte and apical transcript localization in blastoderm embryos. We demonstrate *in vivo* that Egalitarian (Egl) and Bicaudal D (BicD), maternal proteins required for oocyte determination, are selectively recruited by, and co-transported with, localizing transcripts in blastoderm embryos, and that interfering with the activities of Egl and BicD blocks apical localization. We propose that Egl and BicD are core components of a selective dynein motor complex that drives transcript localization in a variety of tissues.

During *Drosophila* oogenesis, specification of the oocyte is associated with selective accumulation of RNA determinants supplied by the neighbouring, interconnecting ovarian nurse cells^{1,2}. Subsequently, deposition of mRNA transcripts at selected sites within the oocyte leads to localized translation of the proteins that establish the prospective embryonic body axes. *gurken* (*grk*) transcripts reside first posteriorly and then anterodorsally, and sequentially establish the anteroposterior and dorsoventral axes^{3–5}. *bicoid* (*bcd*) and *oskar* (*osk*) transcripts localize to the anterior and posterior of the oocyte, respectively, to pattern the anteroposterior body axis^{6–8}.

Asymmetric RNA localization is also evident during zygotic development, especially in the unicellular syncytial blastoderm embryo. At this stage, several transcripts including those of the pair-rule and *wingless* (*wg*) segmentation genes lie exclusively apically of the layer of several thousand peripheral nuclei. Localization of these transcripts seems to be mediated by signals within their 3' untranslated regions (UTRs)^{9–11}, and to be driven on microtubules¹⁰ by the minus-end-directed molecular motor, dynein¹². However, the linkers and other factors that provide the cargo specificity are unknown. Nor is it clear if transcript localization in blastoderm embryos relates to that in other types of cells.

Previously, we reported rapid apical localization of fluorescently labelled *fushi tarazu* (*ftz*) pair-rule transcripts injected into the basal cytoplasm of the cycle 14 blastoderm embryo¹⁰. Although these experiments indicated a requirement for nuclear proteins, more recently we, and others¹², have found that fluorescein labelling compromises the structure of the transcripts, and that pair-rule (*even-skipped*, *hairy* (*h*), *ftz*, *paired* and *runt*) and *wg* transcripts labelled with several other fluorochromes localize apically within 5–8 min without the need for exogenous protein (Fig. 1 and Table 1; see also Methods). Indeed, injected unlabelled transcripts also localize apically (Fig. 1; see also Methods). The protein-free assay retains specificity for apical transport, as transcripts that are normally unlocalized (*Krüppel* (*Kr*), *huckebein*) or enriched in the basal cytoplasm (*string* (*stg*)) are not transported apically and instead diffuse away from the site of injection (Fig. 1 and Table 1).

Shared localization signals and machinery

We used the injection assay to investigate whether any maternal transcripts that localize in the oocyte are recognized by the localiza-

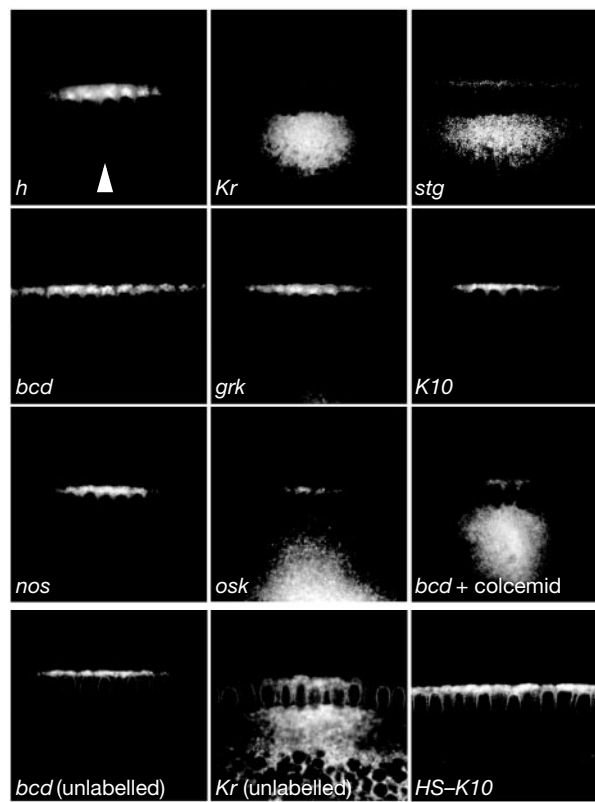


Figure 1 Localization of zygotic and maternal transcripts in blastoderm embryos. Confocal images of representative blastoderm embryos injected with various transcripts. Injected RNA is excluded from the layer of peripheral nuclei. Preinjection with colcemid inhibits localization of injected *bcd* transcripts (right panel, third row). The left and middle panels of the bottom row show apical localization of injected unlabelled *bcd* but not *Kr* transcripts, respectively. Apical localization of endogenously expressed *K10* transcripts (induced by heat shock; *HS-Kr* transcripts do not localize; not shown) is shown in the bottom row, right panel. Transcripts were visualized by fluorescence (top three rows) or by *in situ* hybridization and nuclear staining with Alexa 660-labelled WGA (bottom row). Arrowhead in the top left panel indicates approximate site of injection. Apical is to the top and basal to the bottom in this and all subsequent figures. Scale bar, 50 μ m.

Table 1 Summary of transcript injections

Transcript	Without colcemid	With colcemid	Recruitment of Egl and BicD
<i>even-skipped</i>	85 (40)	ND	ND
<i>ftz</i>	92 (122)	7* (15)	+
<i>h</i>	93 (83)	0 (18)	+
<i>paired</i>	92 (26)	ND	ND
<i>runt</i>	100 (25)	ND	ND
<i>wg</i>	97 (33)	ND	+
<i>huckebein</i>	0 (43)	N/A	ND
<i>Kr</i>	0 (36)	N/A	–
<i>stg</i>	0 (52)	N/A	–
<i>bcd</i>	100 (32)	18* (39)	+
<i>grk</i>	91 (77)	22* (23)	+
<i>K10</i>	100 (63)	20* (25)	+
<i>nos</i>	96 (26)	36* (22)	+
<i>osk</i>	77* (75)	14* (22)	+/-†
<i>bcd4496G → U</i>	22* (60)	ND	–
<i>Kstem5'</i>	5* (22)	N/A	–
<i>Kstem5'3'</i>	100* (28)	ND	ND
<i>Kr–K10TLS</i>	100 (21)	ND	+
<i>stg–K10TLS</i>	100 (39)	ND	+

Numbers indicate the percentage of embryos showing localization; numbers in parentheses indicate the number of embryos. N/A, not applicable; ND, not determined.
* Weak localization (see text and *osk* transcript localization in Fig. 1).
† Weak recruitment, only to localizing transcripts.

tion machinery of blastoderm embryos. We tested five such transcripts (*bcd*, *grk*, *nanos* (*nos*), *osk* and *female sterile (1) K10* (*K10*)), and found that all accumulate in the apical cytoplasm after injection (Fig. 1 and Table 1). With the exception of *osk* transcripts—only a small proportion of which localize apically—the efficiency of localization of these transcripts appears indistinguishable from that of pair-rule transcripts. Maternal transcripts also localize apically when zygotically expressed from endogenous transgenes (Fig. 1, *K10*; *bcd* 3' UTR⁹). Preinjection with colcemid severely inhibits apical localization of the injected maternal transcripts, indicating that their localization in blastoderm embryos, like that of the pair-rule transcripts^{10,12}, is dependent on intact microtubules (Fig. 1 and Table 1).

Further experiments show that the same signals mediate transcript transport during oogenesis and apical localization in blastoderm embryos. We focused on transcripts of the *K10* gene, which localize through a 44-nucleotide region of the 3' UTR (transport/localization sequence; *K10TLS*)¹³—the shortest signal thus far shown to be active during oogenesis. This signal, which is predicted to form a stem-loop structure (Fig. 2a), mediates all aspects of *K10* transcript localization, that is, transport of transcripts from the nurse cells into the oocyte from stage 2 and localization at its anterior pole between stages 8–10 (ref. 13).

We found that the *K10TLS* is sufficient to drive apical localization in blastoderm embryos. Reporter *stg* and *Kr* transcripts into which we inserted the *K10TLS* (*stg–K10TLS* and *Kr–K10TLS*) localize apically in a way that is indistinguishable from pair-rule transcripts (Fig. 2a and Table 1). Moreover, the same regulatory signals are used for transcript localization during oogenesis and in the embryo. A 5-nucleotide transversion in the *K10* transcript that disrupts base pairing of the *K10TLS* stem-loop (Fig. 2b) abolishes all aspects of localization during oogenesis (*Kstem5'*; ref. 13), and prevents *K10* transcripts from localizing in our blastoderm injection assay (Fig. 2b and Table 1). *Kstem5'3'*, in which compensatory mutations restore base pairing in the stem (Fig. 2c), directs weak but significant localization in embryos (Fig. 2c and Table 1). The same signal also partially restores localization during oogenesis¹³. These results suggest that the same machinery is used in both cases.

The common aspect of maternal RNA localization measured in our experiments is unlikely to be transport within the oocyte, because the maternal transcripts tested are distinctly distributed in late stage oocytes by means of different motors and accessory factors². However, all the transcripts—with the possible exception of *grk*¹⁴—are synthesized in adjacent nurse cells and reach the

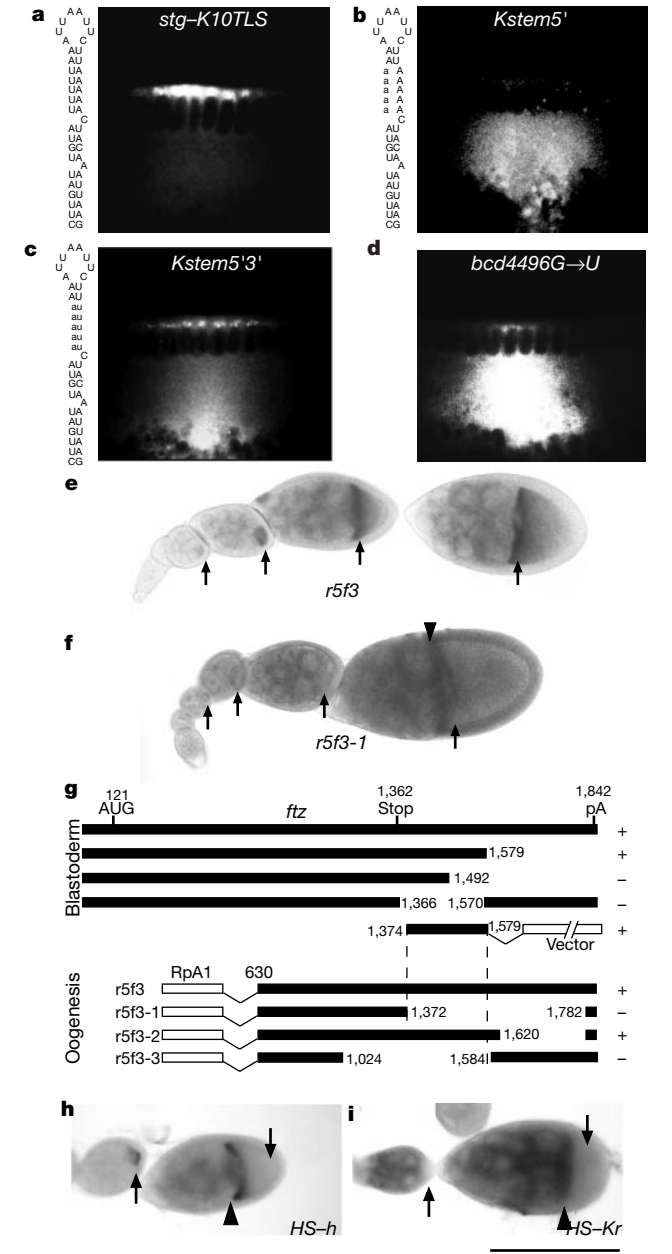


Figure 2 Conservation of RNA localization signals during oogenesis and embryogenesis. **a–c**, Predicted secondary structures (left) and correspondingly injected embryos (right) of the wild-type *K10* transport/localization sequence (*K10TLS*) in the context of a *stg* reporter transcript (**a**), *Kstem5'* in which the primary sequence is altered but the stem-loop is disrupted (**b**) and *Kstem5'3'* in which primary sequence is altered but the stem-loop is restored (**c**). **b** and **c** are in the context of the *K10* 3' UTR. Modified bases in secondary structures are indicated in lower-case letters. **d**, *bcd4496G→U* transcripts in which apical localization is abolished (compare with Fig. 1). Apical localization is efficient in **a** and weak but significant in **c**. **e**, Maternal *ftz* transcripts (*r5f3*) localize within and at the anterior of the oocyte. **f**, *r5f3-1* transcripts that lack the *ftz* 3' UTR do not accumulate specifically during oogenesis. Transcript accumulation anterior to the oldest oocyte is in somatic follicle cells (arrowhead), perhaps because of missing somatic transcript instability elements¹⁶. **g**, *ftz* constructs tested (*n* ≈ 20 embryos, ≈ 100 egg chambers) for transcript localization in blastoderm embryos or during oogenesis. Plus and minus signs indicate presence or absence of apical/oocyte-specific localization, respectively. The minimal signal in blastoderm embryos between positions 1,374 and 1,579 (S.L.B., unpublished observations) is also required for localization in ovaries. The vector tag RNA (1.5-kb fragment of pCR2.1TOPO, Invitrogen; see Methods) is not otherwise able to localize. **h**, **i**, Oocyte-specific localization of heat-shock-induced *h*, but not *Kr*, transcripts that are excluded from the oocyte (arrowhead indicates anterior border of oocyte). Egg chambers are positioned with anterior and earliest stages to the left; oocytes are labelled by arrows. Scale bar: **a–d**, 50 μm; **e**, **f**, **h**, **i**, 150 μm.

oocyte by transport along microtubules². To test whether this process is analogous to apical localization in blastoderm embryos, we injected a *bcd* transcript containing a single nucleotide change (4496G→U), which prevents early oocyte-specific transport (stages 4–6) without disrupting later (stage 6 onwards) import of transcripts into the oocyte or their subsequent accumulation at the anterior cortex¹⁵. This mutation inhibits apical *bcd* localization in blastoderm embryos (Fig. 2d and Table 1), suggesting that transcripts localize in our injection assay through the same machinery that transports transcripts into the early oocyte.

This proposal is supported by our finding that pair-rule transcripts accumulate in the early oocyte if synthesized ectopically during oogenesis. *r5f3* females express a hybrid transcript containing a portion of the *ftz* coding sequence and the entire 3' UTR under the control of the constitutively active *RpA1* promoter¹⁶. *r5f3* transcripts accumulate specifically in the oocyte from stage 3 (Fig. 2e), and concentrate at the anterior cortex of the oocyte between stages 8 and 10B, after which they become distributed throughout the oocyte. This pattern of localization is indistinguishable from that of *K10* transcripts and closely follows the distribution of the minus ends of microtubules¹⁷. Localization of *r5f3* is dependent on an intact microtubule cytoskeleton, as it is inhibited by prior treatment with colchicine¹⁸ (not shown). A hybrid *ftz* transcript (*r5f3-1*) lacking the 3' UTR, and therefore the signal for apical localization in blastoderm embryos⁹, is retained in the nurse cells and not transported to the oocyte (Fig. 2f).

These results indicate that blastoderm localization signals can drive transcript transport during oogenesis. This view is supported by more detailed analysis of maternally expressed pair-rule transcripts. Our injection assay reveals a minimum region between positions 1,374 and 1,579 in *ftz* that is necessary and sufficient for localization in blastoderm embryos (Fig. 2g and S.L.B., unpublished results). A similar region of *ftz* seems to be required for localization of transcripts into the oocyte (Fig. 2g). Furthermore, *h* (Fig. 2h) and *runt* (data not shown) transcripts, driven maternally by the *Hsp70* promoter, also accumulate specifically in the oocyte and later reside at its anterior cortex, whereas *Kr* or truncated *h* transcripts lacking most of the 3' UTR (*HSH21*; ref. 19) fail to localize either in blastoderm embryos⁹ or during oogenesis (Fig. 2i and data not shown).

Recruitment of Egl and BicD to localizing transcripts

The above data suggest that components of the blastoderm localization machinery are also likely to function in RNA transport into the early oocyte. Genetic screens for maternal mutations that affect formation of the embryonic axis have identified *egl* and *BicD* as genes required for oocyte differentiation and for specific RNA accumulation in the oocyte^{20–25}. However, their exact activities are uncertain. BicD includes multiple heptad repeats^{26,27}, which may mediate oligomerization and interactions with other proteins; Egl includes a domain shared with 3'–5' exonucleases²⁸. During oogenesis, these two proteins form complexes together²⁴ and colocalize at the minus ends of microtubules^{24,29}. The integrity of the microtubule cytoskeleton is defective in *egl* and *BicD* mutants, which has been proposed to explain subsequent defects in RNA localization^{23,29}. Alternatively, Egl and BicD might act directly in RNA transport^{24,30}. However, evidence that distinguishes between these two possibilities is lacking.

We first examined whether Egl and BicD are present in early embryos. Both proteins are supplied maternally to the embryo. They are noticeably enriched apically to the nuclei at blastoderm stages (Fig. 3a) where they colocalize with dynein heavy chain (Dhc; Fig. 3a)—a component of the motor associated with apical transcript transport¹². Nevertheless, a large proportion of both of the proteins is present in the basal cytoplasm.

We next tested whether endogenous Egl and BicD can associate with injected localizing transcripts, as might be expected if they are

components of the RNA localization machinery. Injection of *h* transcripts leads to marked enrichment of Egl and BicD protein levels at the sites of RNA localization (Fig. 3b). Similar results are found on injection of the other tested maternal and zygotic localizing transcripts (*ftz*, *bcd*, *grk*, *K10*, *nos*, *osk* and *wg*) (Fig. 3b and Table 1). Both proteins accumulate basally at the site of injection within 1–2 min (Fig. 3b). Protein recruitment is not inhibited in embryos preincubated with colcemid, showing that it is not dependent on intact microtubules (Fig. 3b). Thus, the proteins are recruited locally before transport and are transported together apically with transcripts.

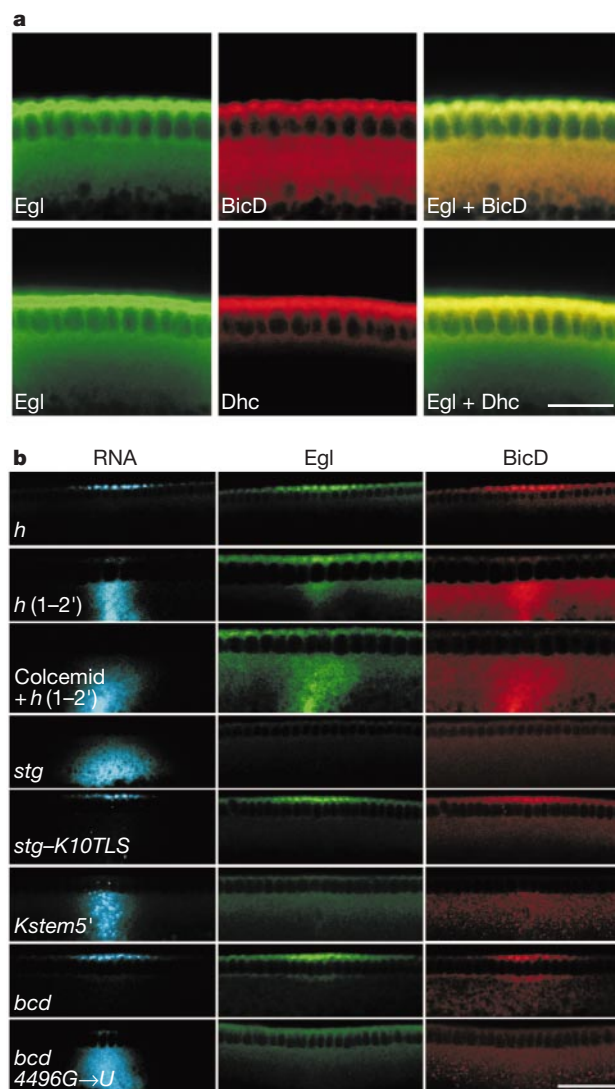


Figure 3 Colocalization of Egl, BicD and Dhc proteins and recruitment of Egl and BicD to localizing transcripts. **a**, Immunostaining of cycle 14 blastoderm embryos for Egl and BicD (top panel, green and red, respectively) and for Egl and Dhc (bottom panel, green and red, respectively). All three proteins colocalize apically of the nuclei. Merged images are on the right. **b**, Egl (green) and BicD (red) protein distribution in embryos injected with localizing (*h*, *stg-K10TLS*, *bcd*) or non-localizing (*stg*, *Kstem5'*, *bcd4496G→U*) RNAs (blue), or preinjected with colcemid 10 min before RNA injection. There are increased levels of Egl and BicD at the sites of RNA localization. Embryos were fixed 5–8 min after RNA injection, except in the second and third rows when they were fixed 1–2 min after injection. Elevated concentrations of transcripts were used in these experiments to emphasize protein enrichment over injection site (Methods). BicD and Egl are also recruited to unlabelled localizing transcripts (not shown). Scale bar: **a**, 40 μm; **b**, 100 μm (except for the 2nd and 3rd rows where it represents 60 μm).

Interaction of injected transcripts with Egl/BicD is mediated by intact localization signals: we detected protein recruitment to *Kr-K10TSL* and *stg-K10TSL*, but not to *Kr*, *stg*, *Kstem5'* and *bcd4496G→U* (Fig. 3b and Table 1), or to a *h* transcript containing a 21-base-pair (bp) deletion within the localization signal that abolishes localization (S.L.B. and D.I.-H., unpublished data). When localization is weak, we only detected recruitment of Egl and BicD by transcripts that have localized apically (for example, *osk*; data not shown). The above results suggest that only transcripts that bind Egl/BicD can be transported apically.

Egl and BicD are required for apical RNA localization

We next examined whether BicD and Egl are required for apical localization in blastoderm embryos. Strong *BicD* alleles block oogenesis early^{21,25–27}, and weaker mutant mothers that lay fertilized eggs (*BicD*^{HA40}/*BicD*^{R26} and *BicD*^{H3}/*BicD*^{R26}; see Methods³¹) retain sufficient BicD activity for a normal apical distribution of endogenous pair-rule transcripts (data not shown). However, the reduced BicD activity in these embryos no longer supports efficient transport of injected transcripts: 62% of *BicD*^{HA40}/*BicD*^{R26} and 73% of *BicD*^{H3}/*BicD*^{R26} embryos show no or weak localization 5–8 min after injection, compared with 10% of wild-type embryos (Fig. 4b).

Moreover, we show that an antibody against BicD blocks RNA transport. Preinjection into the basal cytoplasm of anti-BicD antibody 4C2 (ref. 21) strongly inhibits the localization of injected *h* (Fig. 4a, b), *ftz*, *grk* and *stg-K10TSL* (data not shown) transcripts in 70–75% of embryos, whereas injection buffer alone has no effect on localization (Fig. 4a, b). Control anti-Staufen (Stau) and anti-Orb antibodies do not affect localization (Fig. 4b). The microtubule cytoskeleton is not obviously affected by the brief (≤20 min) antibody treatment (Fig. 4a), indicating that the effects on RNA transport are probably direct. Injection of anti-BicD antibody (but not injection buffer, anti-Orb or anti-Stau; Fig. 4a and not shown) prevents apical localization of endogenous pair-rule transcripts, also leading to anteroposterior smearing of their distribution (Fig. 4a). Thus, apical transcript localization seems to be important in restricting the range of activity of pair-rule genes, and allowing their combinatorial control of *Drosophila* segmentation.

To confirm that the antibody acts specifically on BicD, we assessed its blocking activity in embryos from mutant mothers (*BicD*^{HA40}) that only express 6% of wild-type BicD protein levels³¹ (Methods). This level of BicD production is sufficient for full maternal and zygotic function³¹, and permits normal apical localization of endogenous pair-rule transcripts in embryos (data not shown), although localization of injected transcripts is impaired in 32% of embryos (Fig. 4b). Notably, *BicD*^{HA40} embryos are sensitive to dilutions of anti-BicD antibody that have little effect on wild-type embryos. For example, a 1:4 dilution of antibody inhibits the localization of injected transcripts in 94% of *BicD*^{HA40} embryos compared with 23% of wild-type embryos (Fig. 4b). This demonstrates that the antibody inhibits localization by interacting with BicD, and confirms that BicD is required for transcript localization in blastoderm embryos.

Injecting blastoderm embryos with anti-Egl²⁴ also inhibits apical localization of both exogenous and endogenous pair-rule transcripts, without overtly disrupting the microtubule network (Fig. 4a, b). Moreover, its effect is more potent in embryos from mothers containing only a single copy of the *egl* gene (*Df(2R) bw-S46/CyO*; Fig. 4b), indicating that the antibody disrupts RNA localization by inhibiting the activity of Egl. Egl and BicD are probably also involved in transporting other cargoes. The arrangement of peripheral nuclei is disrupted after injection of antibodies to either of the two proteins, consistent with previous data showing a requirement for BicD in nuclear migration in eye imaginal disc cells³². Embryos injected with either antibody undergo abnormal morphogenesis (data not shown), which is also indicative of Egl and BicD transporting additional cargoes.

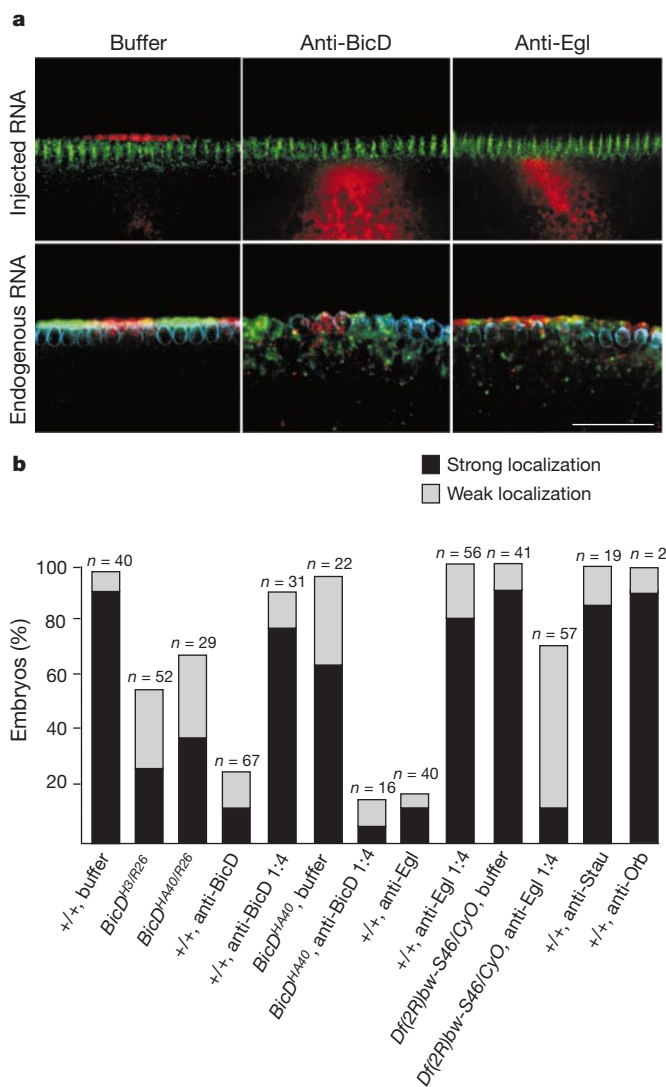


Figure 4 Requirement for BicD and Egl for apical transcript localization in the blastoderm. **a**, Distribution of injected (*h* (red)) and two endogenous (*ftz* (red) and *h* (green)) transcripts after preinjection with injection buffer alone (buffer) or antibodies against BicD or Egl. Both antibodies block apical localization and lead to accumulation of injected and endogenous RNA in the basal cytoplasm. Top, β -tubulin (green) distribution (enriched between the nuclei) is not obviously affected by either antibody. Bottom, nuclear envelopes are stained with Alexa 660-conjugated WGA (blue). In control embryos, pair-rule transcripts lie in circumferential stripes 3–4 nuclei wide. Pair-rule transcripts have a broader distribution after antibody injection. Nuclear organization is disrupted after antibody injection. Scale bar, 50 μ m. **b**, Percentage of embryos displaying strong, weak or no localization of injected *h* transcripts (see Methods for classification). Localization is impaired in embryos from mothers with reduced BicD levels (*BicD*^{H3}/*BicD*^{R26}, *BicD*^{HA40}/*BicD*^{R26}) or after injection with anti-BicD or anti-Egl. Diluted antibodies block localization more effectively in embryos with reduced BicD (*BicD*^{HA40}) or Egl (*Df(2R)bw-S46/CyO*) levels. Results are the total of two–three separate experiments. *n* is the number of embryos injected. Similar results were found with injected *ftz* transcripts (data not shown).

Discussion

Our results indicate that Egl and BicD are principal elements of a complex that transports RNA in blastoderm embryos. Egl and BicD appear to be present as pools of excess cytoplasmic protein that associate selectively with localizing transcripts and are transported together apically. Protein recruitment occurs before transport and does not require microtubule integrity; rather, transport depends on Egl and BicD activity. Egl and BicD probably act directly to mediate RNA transport associated with establishment and

maintenance of the oocyte. Thus, mutant transcripts that are defective in export from nurse cells into the oocyte fail to recruit Egl or BicD in blastoderm embryos. *grk* transcripts are also recognized by the Egl–BicD–microtubule transport pathway, which is consistent with the hypothesis that nurse cells are a source of these transcripts for the early oocyte³³ and that they do not derive exclusively from the oocyte nucleus¹⁴.

Egl/BicD is enriched at sites of RNA localization in both blastoderm embryos and oocytes^{21,24}, presumably as the consequence of protein/RNA co-transport. The complex may have an additional role in anchoring transcripts at their destination. Alternatively, maintenance of localized transcripts might not depend on an independent anchorage step, but result from sustained minus-end-directed transport.

The structural basis of how the transport machinery and RNA signals recognize each other is unclear. The shortest signal defined to date, the *K10* transport/localization sequence¹³, probably relies on both primary and secondary structure. Thus, mutating bases in the stem (*Kstem5'*) inactivates the signal, and compensatory mutations that restore base pairing in the stem (*Kstem5'3'*) reactivates the signal. However, the *Kstem5'3'* signal is only partially active, indicating that primary sequence and possibly tertiary structure are also important. Nor were we able to identify shared structural features in several maternal and zygotic localization signals (S.L.B. and D.I.-H., unpublished data). This could be due to promiscuous or multiple adapter proteins, or because the motor protein complex allows alternative RNA contacts. Egl or BicD may contribute directly to determining RNA selectivity. Neither includes a well-characterised RNA-binding motif, but Egl includes a domain found in a variety of nucleic-acid-recognizing proteins (ref. 28 and J. Sgouros, personal communication). Other components of the complex may also contribute to selective RNA recognition in blastoderm embryos. However, none of the proteins currently implicated in localizing maternal transcripts are likely candidates for such adapters, being absent in blastoderm embryos (Orb³⁴, Swallow (Swa)³⁵, Exuperantia (Exu)^{36,37}), not required for early transport of transcripts into the oocyte (Stau, Exu, Swa)³⁸, or not recruited to localizing pair-rule transcripts (Stau; S. Lall, S.L.B. and D.I.-H., unpublished data).

Dhc, Egl and BicD have markedly similar distributions during oogenesis and in blastoderm embryos, and seem to function together in specifying oocyte identity³². We propose that an Egl/BicD complex links specific RNAs to dynein and the microtubules. The same machinery may operate elsewhere in *Drosophila*. For example, *inscuteable* transcripts, which localize asymmetrically in neuroblasts³⁹, also localize apically when injected into blastoderm embryos (J. R. Hughes, S.L.B. and D.I.-H., unpublished data). Indeed, germline transcripts localize apically when expressed in follicle cells⁴⁰. Egl and BicD homologues have been identified in *Caenorhabditis elegans* (GenBank accession numbers U58745 and U70848 for Egl and BicD, respectively) and mammals (BicD⁴¹), and might comprise part of an evolutionarily conserved cytoskeletal system for transporting transcripts and other cargoes. □

Methods

Fly culture

Wild-type flies are of the strain Oregon-R. The *r5f3*, *r5f3-1*, *r5f3-2* and *r5f3-3* transgenes include the *RpA1* promoter and 0.2 kb of its 5'-transcribed sequences¹⁶. Full genotypes of *BicD* mutant flies are: *BicD^{HA40}*, (*P{BicD^{HA40}}*; *BicD^{RS}/Df(2L)TW119*); *BicD^{HA40}/BicD^{R26}*, (*P{BicD^{HA40}}*; *BicD^{R26}/Df(2L)TW119*); *BicD^{H3}/BicD^{R26}*, (*P{BicD^{H3}}*; *BicD^{R26}/Df(2L)TW119*). *BicD^{RS}*, a protein null, is described in ref. 25. *BicD^{R26}* (ref. 20), an antimorphic allele, *P{BicD^{H3}}* and *P{BicD^{HA40}}*, which expresses 12% as much BicD as the wild-type gene (6% of diploid production), are described in ref. 31. Transgenes containing the heat-inducible *Hsp70* promoter were used to drive ectopic expression of *h* (using *HSH33* and *HSH21* lines¹⁹), *Kr* (*HS-Kr*; G. Struhl, unpublished data) and *K10* (*HS-K10*; ref. 40).

For heat-shock-induction in egg chambers (nurse and follicle cells, but not oocytes),

adult females were incubated at 36.8 °C for 1 h and incubated for 6 h at 25 °C before fixation⁴⁰. Cycle 13–14 blastoderm embryos were heat-shocked at 36.0 °C for 15 min and incubated at 25 °C for 15 min before fixation.

Fluorescent RNA synthesis

Linearized plasmid template was transcribed in a solution containing 0.4 mM ATP, 0.4 mM CTP, 0.36 mM UTP, 0.04 mM Cy3-, Cy5- (NEN Life Sciences) or Alexa-488 (Molecular Probes) UTP, 0.12 mM GTP and 0.3 mM 7mG(5')pppG cap analogue (Stratagene) using SP6, T3 or T7 polymerases (Roche) according to the manufacturer's instructions. UTP concentration was 0.4 mM for synthesis of unlabelled transcripts. RNA was treated with DNase I, extracted with phenol/chloroform, spun through a mini Quick Spin G50 column (Roche) to remove unincorporated nucleotides, and precipitated with NH₄OAc/EtOH. Fluorescent RNAs typically contained 1 fluorochrome/250 nucleotides. In contrast to fluorescein-labelled transcripts¹⁰, Cy3- or Cy5- or Alexa 488-labelled RNAs localize apically without incubation with nuclear factors. Transcripts labelled with Alexa 488-UTP have a propensity to form large particles, which are nevertheless capable of localizing. Unlabelled *h*, *bcd* and *nos* transcripts (detected by *in situ* hybridization) also localize apically.

Injections and histochemistry

Protein-free RNA was injected into embryos together with 1 µg µl⁻¹ cycloheximide to inhibit transcript degradation, as described¹⁰, although similar results were achieved when cycloheximide was omitted. In all of the experiments 200 ng µl⁻¹ RNA was injected, with the exception of experiments addressing Egl and BicD recruitment when RNA concentration was 1 µg µl⁻¹. Typically, 15–30 blastoderm embryos were injected in a single experiment. In some experiments, injection buffer, colcemid (20 ng µl⁻¹; final intracellular concentration is ~50 times less), anti-BicD antibody (mouse monoclonal 4C2 (ref. 21)), anti-Egl antibody (rabbit polyclonal²⁴), anti-Orb antibody (mouse monoclonal 6H4 (ref. 42)) or anti-Stau antibody (rabbit polyclonal⁴³) was injected into embryos 10 min before RNA injection. Unless stated otherwise, embryos were fixed 10 min after injection of the last embryo with RNA (~13 min after injection of the first embryo) in heptane saturated with 37% formaldehyde for 10 min, before hand-peeling. For detection of endogenous pair-rule transcripts, embryos were injected with injection buffer or antibody 25–30 min before fixation for 5 min in heptane saturated with 37% formaldehyde. This longer incubation increased the likelihood that we were not visualizing transcripts that were synthesized and transported before antibody injection.

For immunofluorescence, injected embryos were fixed for 5 min in heptane saturated with 37% formaldehyde, hand-peeled and washed in PBS/0.1% Triton X-100. Embryos were incubated overnight at 4 °C with mouse monoclonal anti-BicD antibody 1B11 (1:20; ref. 21), rabbit polyclonal anti-Egl antibody (1:2,000), mouse monoclonal anti-Dhc antibody (1:20; ref. 44) or mouse anti-β-tubulin antibody (1:100; Sigma). Secondary antibodies (Molecular Probes) were conjugated to Alexa 488 or Alexa 594. Mounted embryos were imaged with a Zeiss LSM 510 confocal microscope, and the proportion of injected RNA in the apical and basal cytoplasm was estimated using Zeiss LSM 510 software. Localization was designated as strong (most of the RNA in the apical cytoplasm, for example pair-rule transcripts), weak (most of the RNA remains basal but there are some apical caps of RNA, for example *osk* transcripts) or unlocalized (no apical concentration of RNA, for example *Kr* transcripts).

In situ detection of transcripts during oogenesis was performed essentially as described⁴⁵ except that we used labelled RNA probes. *In situ* hybridization of embryos was performed using fluorescein- and Cy3-tyramides (NEN) followed by Alexa 660-wheat germ agglutinin (WGA; Molecular Probes) labelling of nuclear pore complexes¹².

Plasmid construction

All transcripts were derived from full-length complementary DNAs, with the exception of *K10* and its derivatives, which was a *SalI*–*PstI* fragment corresponding to the entire 3' UTR and a 860-bp portion of the 3' genomic sequences. For production of *stg*–*K10TSL* and *Kr*–*K10TSL* constructs, a fragment corresponding to the entire 44-nucleotide *K10TSL* (residues 52–95; GenBank accession number U36587) flanked by artificial *BglII* and *BamHI* sites (gift of R. S. Cohen) was blunted and inserted into the *StuI* site of *stg* (position 1,727; M24909) or the *NruI* site of *Kr* (position 1,289; X03414), respectively. The *bcd4496G*→*U* mutation was introduced into full-length *bcd* cDNA using the Quickchange site-directed mutagenesis kit (Stratagene). Modified *fz* constructs for mapping the blastoderm localization signal were generated by PCR or by Quickchange mutagenesis. The pCR2.1TOPO vector RNA is derived from a 1.5-kb *EcoRI*/*NcoI* fragment.

Received 10 July; accepted 12 September 2001.

1. St Johnston, D. The intracellular localization of messenger RNAs. *Cell* **81**, 161–170 (1995).
2. Bashirullah, A., Cooperstock, R. L. & Lipshitz, H. D. RNA localization in development. *Ann. Rev. Biochem.* **67**, 335–394 (1998).
3. Neuman-Silberberg, F. S. & Schüpbach, T. The *Drosophila* dorsoventral patterning gene *gurken* produces a dorsally localized RNA and encodes a TGFα-like protein. *Cell* **75**, 165–174 (1993).
4. Gonzalez-Reyes, A., Elliott, H. & St Johnston, D. Polarization of both major body axes in *Drosophila* by *gurken*-torpedo signalling. *Nature* **375**, 654–658 (1995).
5. Roth, S., Neuman-Silberberg, F. S., Barcelo, G. & Schüpbach, T. *cornichon* and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in *Drosophila*. *Cell* **81**, 967–978 (1995).
6. Berleth, T. et al. The role of localisation of *bicoid* RNA in organising the anterior pattern of the *Drosophila* embryo. *EMBO J.* **7**, 1749–1756 (1988).

7. Ephrussi, A., Dickinson, L. K. & Lehmann, R. *oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. *Cell* **66**, 37–50 (1991).
8. Kim-Ha, J., Smith, J. L. & MacDonald, P. M. *oskar* messenger-RNA is localized to the posterior pole of the *Drosophila* oocyte. *Cell* **66**, 23–34 (1991).
9. Davis, I. & Ish-Horowitz, D. Apical localisation of pair-rule transcripts requires 3' sequences and limits protein diffusion in the *Drosophila* blastoderm embryo. *Cell* **67**, 927–940 (1991).
10. Lall, S., Francis-Lang, H., Norvell, A., Schüpbach, T. & Ish-Horowitz, D. Squid hnRNP protein promotes apical cytoplasmic transport and localisation of *Drosophila* pair-rule transcripts. *Cell* **98**, 171–180 (1999).
11. Simmonds, A., DosSantos, G., Livne-Bar, I. & Krause, H. Apical localization of *wingless* transcripts is required for Wingless signaling. *Cell* **105**, 197–207 (2001).
12. Wilkie, G. S. & Davis, I. *Drosophila wingless* and pair-rule transcripts localise apically by dynein mediated transport of RNA particles. *Cell* **105**, 209–219 (2001).
13. Serano, T. L. & Cohen, R. S. A small predicted stem-loop structure mediates oocyte localization of *Drosophila K10* mRNA. *Development* **121**, 3809–3818 (1995).
14. Saunders, C. & Cohen, R. S. The role of oocyte transcription, the 5'UTR, and translation repression and derepression in *Drosophila gurken* mRNA and protein localization. *Mol. Cell* **3**, 43–54 (1999).
15. Macdonald, P. M. & Kerr, K. Redundant RNA recognition events in *bicoid* mRNA localization. *RNA* **3**, 1413–1420 (1997).
16. Riedl, A. & Jacobs-Lorena, M. Determinants of *Drosophila fushi tarazu* mRNA instability. *Mol. Cell Biol.* **16**, 3047–3053 (1996).
17. Theurkauf, W. E. Microtubules and cytoplasm organization during *Drosophila* oogenesis. *Dev. Biol.* **165**, 352–360 (1994).
18. Pokrywka, N. J. & Stephenson, E. C. Microtubules mediate the localization of *bicoid* RNA during *Drosophila* oogenesis. *Development* **113**, 55–66 (1991).
19. Ish-Horowitz, D. & Pinchin, S. M. Pattern abnormalities induced by ectopic expression of the *Drosophila* gene *hairy* are associated with repression of *fushi tarazu* transcription. *Cell* **51**, 405–415 (1987).
20. Mohler, J. & Wieschaus, E. F. Dominant maternal-effect mutations of *Drosophila melanogaster* causing the production of double-abdomen embryos. *Genetics* **112**, 803–822 (1986).
21. Suter, B. & Steward, R. Requirement for phosphorylation and localization of the Bicaudal-D protein in *Drosophila* oocyte differentiation. *Cell* **67**, 917–926 (1991).
22. Schüpbach, T. & Wieschaus, E. Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. *Genetics* **129**, 1119–1136 (1991).
23. Theurkauf, W. E., Alberts, B. M., Jan, Y. N. & Jongens, T. A. A central role for microtubules in the differentiation of *Drosophila* oocytes. *Development* **118**, 1169–1180 (1993).
24. Mach, J. M. & Lehmann, R. An Egalitarian–BicaudalD complex is essential for oocyte specification and axis determination in *Drosophila*. *Genes Dev.* **11**, 423–435 (1997).
25. Ran, B., Bopp, R. & Suter, B. Null alleles reveal novel requirements for *Bic-D* during *Drosophila* oogenesis and zygotic development. *Development* **120**, 1233–1242 (1994).
26. Wharton, R. P. & Struhl, G. Structure of the *Drosophila Bicaudal-D* protein and its role in localizing the posterior determinant *nanos*. *Cell* **59**, 881–892 (1989).
27. Suter, B., Romberg, L. M. & Steward, R. *Bicaudal-D*, a *Drosophila* gene involved in developmental asymmetry-localized transcript accumulation in ovaries and sequence similarity to myosin heavy-chain tail domains. *Genes Dev.* **3**, 1957–1968 (1989).
28. Moser, M. J., Holley, W. R., Chatterjee, A. & Mian, I. S. The proofreading domain of *Escherichia coli* DNA polymerase I and other DNA and/or RNA exonuclease domains. *Nucleic Acids Res.* **25**, 5110–5118 (1997).
29. Oh, J. & Steward, R. *Bicaudal-D* is essential for egg chamber formation and cytoskeletal organization in *Drosophila* oogenesis. *Dev. Biol.* **232**, 91–104 (2001).
30. Swan, A. & Suter, B. Role of Bicaudal-D in patterning the *Drosophila* egg chamber in mid-oogenesis. *Development* **122**, 3577–3586 (1996).
31. Oh, J., Baksa, K. & Steward, R. Functional domains of the *Drosophila* Bicaudal-D protein. *Genetics* **154**, 713–724 (2000).
32. Swan, A., Nguyen, T. & Suter, B. *Drosophila* Lissencephaly-1 functions with Bic-D and dynein in oocyte determination and nuclear positioning. *Nature Cell Biol.* **1**, 444–449 (1999).
33. Thio, G. L., Ray, R. P., Barcelo, G. & Schüpbach, T. Localization of *gurken* RNA in *Drosophila* oogenesis requires elements in the 5' and 3' regions of the transcript. *Dev. Biol.* **221**, 435–446 (2000).
34. Lantz, V. & Schedl, P. Multiple *cis*-acting targeting sequences are required for *orb* mRNA localization during *Drosophila* oogenesis. *Mol. Cell Biol.* **14**, 2235–2242 (1994).
35. Schnorrer, F., Bohmann, K. & Nusslein-Volhard, C. The molecular motor dynein is involved in targeting Swallow and *bicoid* RNA to the anterior pole of *Drosophila* oocytes. *Nature Cell Biol.* **2**, 185–190 (2000).
36. Macdonald, P. M., Luk, S. K. & Kilpatrick, M. Protein encoded by the *exuperantia* gene is concentrated at sites of *bicoid* mRNA accumulation in *Drosophila* nurse cells but not in oocytes or embryos. *Genes Dev.* **5**, 2455–2466 (1991).
37. Marcey, D., Watkins, W. S. & Hazelrigg, T. The temporal and spatial distribution pattern of maternal Exuperantia protein: evidence for a role in establishment but not maintenance of *bicoid* mRNA localization. *EMBO J.* **10**, 4259–4266 (1991).
38. St Johnston, D., Driever, W., Berleth, T., Richstein, S. & Nüsslein-Volhard, C. Multiple steps in the localization of *bicoid* RNA to the anterior pole of the *Drosophila* oocyte. *Development (Suppl.)* **107**, 13–19 (1989).
39. Li, P., Yang, X., Wasser, M., Cai, Y. & Chia, W. Inscuteable and Staufen mediate asymmetric localization and segregation of *prospero* RNA during *Drosophila* neuroblast cell divisions. *Cell* **90**, 437–447 (1997).
40. Karlin-Meginness, M., Serano, T. L. & Cohen, R. S. Comparative analysis of the kinetics and dynamics of *K10*, *bicoid*, and *oskar* mRNA localization in the *Drosophila* oocyte. *Dev. Genet.* **19**, 238–248 (1996).
41. Baens, M. & Marynen, P. A human homologue (*BICD1*) of the *Drosophila Bicaudal-D* gene. *Genomics* **45**, 601–606 (1997).
42. Lantz, V., Chang, J. S., Horabin, J. I., Bopp, D. & Schedl, P. The *Drosophila orb* RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. *Genes Dev.* **8**, 598–613 (1994).
43. St Johnston, D., Beuchle, D. & Nüsslein-Volhard, C. *staufer*, a gene required to localize maternal RNAs in the *Drosophila* egg. *Cell* **66**, 51–63 (1991).
44. Sharp, D. J. *et al.* Functional coordination of three mitotic motors in *Drosophila* embryos. *Mol. Biol. Cell* **11**, 241–253 (2000).
45. Tautz, D. & Pfeifle, C. A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81–85 (1989).

Acknowledgements

We are grateful to I. Davis, N. MacDougall, G. Wilkie, D. Baker, A. Flament and J. Hughes for discussions; R. Lehmann, R. Steward, D. Sharp, B. Suter, M. Jacobs-Lorena, D. St Johnston, G. Dollar, L. Gavis, B. Cohen and Developmental Studies Hybridoma Bank for providing reagents; and many colleagues for comments on the manuscript. This work was supported by the Imperial Cancer Research Fund and a fellowship from the Royal Commission for the Exhibition of 1851 (to S.L.B.).

Correspondence and requests for materials should be addressed to D.I.-H. (e-mail: d.horowicz@icrf.icnet.uk).

Direct detection of a microlens in the Milky Way

C. Alcock^{*†‡}, R. A. Allsman[§], D. R. Alves^{||}, T. S. Axelrod[¶], A. C. Becker[#], D. P. Bennett[†], K. H. Cook^{*†}, A. J. Drake^{*}, K. C. Freeman[¶], M. Geha^{**}, K. Griest^{†‡}, S. C. Keller^{*}, M. J. Lehner[‡], S. L. Marshall^{*}, D. Minniti^{‡‡}, C. A. Nelson^{*||}, B. A. Peterson[¶], P. Popowski^{*}, M. R. Pratt^{§§}, P. J. Quinn^{|||}, C. W. Stubbs^{†§§}, W. Sutherland^{¶¶}, A. B. Tomaney^{§§}, T. Vandehei^{††} & D. Welch^{##}

^{*} Lawrence Livermore National Laboratory, Livermore, California 94550, USA

[†] Center for Particle Astrophysics, University of California, Berkeley, California 94720, USA

[‡] Department of Physics and Astronomy, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6396, USA

[§] Supercomputing Facility, Australian National University, Canberra, Australian Capital Territory 0200, Australia

^{||} Space Telescope Science Institute, 3700 San Martin Drive, Baltimore, Maryland 21218, USA

[¶] Research School of Astronomy and Astrophysics, Mount Stromlo Observatory, Cotter Road, Weston, Australian Capital Territory 2611, Australia

[#] Bell Laboratories, Lucent Technologies, 600 Mountain Avenue, Murray Hill, New Jersey 07974, USA

^{*} Department of Physics, University of Notre Dame, Indiana 46556, USA

^{**} Department of Astronomy and Astrophysics, University of California, Santa Cruz, California 95064, USA

^{††} Department of Physics, University of California, San Diego, California 92039, USA

^{‡‡} Depto. de Astronomia, P. Universidad Catolica, Casilla 104, Santiago 22, Chile

^{§§} Departments of Astronomy and Physics, University of Washington, Seattle, Washington 98195, USA

^{|||} European Southern Observatory, Karl Schwarzschild Str. 2, D-85748 Garching bei München, Germany

^{¶¶} Department of Physics, University of Oxford, Oxford OX1 3RH, UK

^{##} Department of Physics and Astronomy, McMaster University, Hamilton, Ontario, Canada, L8S 4M1

The nature of dark matter remains mysterious, with luminous material accounting for at most ~ 25 per cent of the baryons in the Universe^{1,2}. We accordingly undertook a survey looking for the microlensing of stars in the Large Magellanic Cloud (LMC) to determine the fraction of Galactic dark matter contained in massive compact halo objects (MACHOs). The presence of the dark matter would be revealed by gravitational lensing of the light from an LMC star as the foreground dark matter moves across the line of sight. The duration of the lensing event is the key observable parameter, but gives non-unique solutions when attempting to estimate the mass, distance and transverse velocity of the lens. The survey results to date indicate that between 8 and 50 per cent of the baryonic mass of the Galactic halo is in the form of MACHOs (ref. 3), but removing the degeneracy by identifying a lensing object would tighten the constraints on the mass in MACHOs. Here we report a direct image of a microlens, revealing it to be a nearby low-mass star in the disk of the Milky Way. This is consistent with the expected frequency of nearby stars acting as lenses, and demonstrates a direct determination of a lens mass from a microlensing event. Complete solutions such as this for halo microlensing events will probe directly the nature of the MACHOs.

The MACHO project has reported^{3,4} microlensing events in the LMC. We have now performed follow-up observations, obtaining images (from the Hubble Space Telescope, HST, Wide Field Planetary Camera 2) of fields containing the source stars of many of these microlensing events. A Planetary Camera image of the LMC-5 system was taken on 13 May 1999 with total exposure times of 1,600 s, 800 s and 1,000 s in the F555W (V), F675 (R) and F814W (I)

bands. This image was taken 6.3 years after the peak of the microlensing event on 5 February 1993. The pixel size of the Planetary Camera ($0.046''$) was sufficient to resolve the microlensing system, and show it to be composed of a faint, red object displaced by $0.134''$ from the centre of an LMC main-sequence star that, on the basis of previous analysis⁴, is the source star of this event (Fig. 1a). Further analysis of the HST data, described here, combined with the results of a fit to the microlensing light curve suggests that the faint, red object is the lens (Fig. 1b). This is consistent with previous suggestions^{5,6} that this event is the result of lensing by a faint Milky Way star. We note that the detection of one such event in our current sample of 13–18 events is completely consistent with the expected number of Milky Way events³ associated with known populations. We have completed HST analysis of 17 of these events, and find no other plausible candidates for lensing by Milky Way stars.

The chance placement of the source star near a foreground object in our image is very low. The density of stars with V-band magnitude $V < 26$ in the LMC-5 frame is $\rho = 1.8 \times 10^{-3}$ per pixel². The frequency of red objects with $V < 26$ and $(V - I) > 2.6$ in all our LMC Planetary Camera image⁴ is 8.8×10^{-4} . Thus the chance superposition of a red object within $0.5''$ of the source star is about 1 in 10,000. Therefore, we assume that

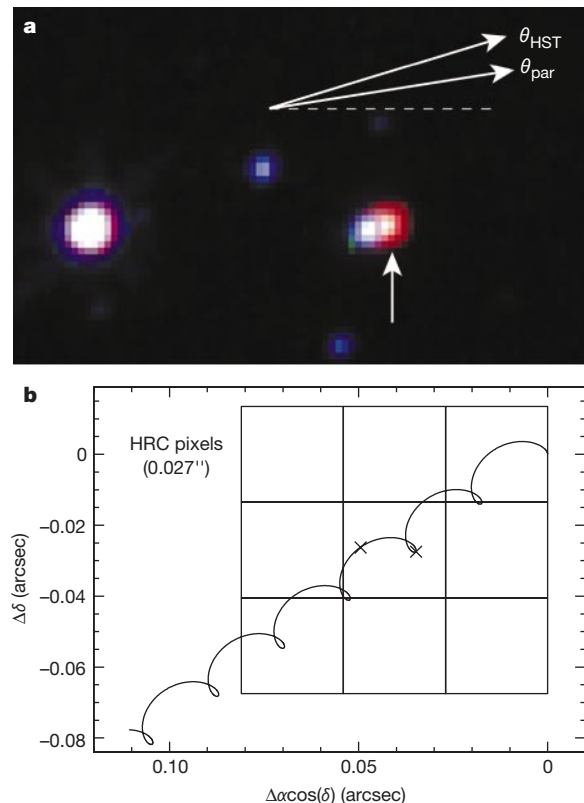


Figure 1 Image and model of the LMC-5 system. **a**, A three-colour composite image, made up of the WPC2 V-, R- and I-band images of LMC-5 obtained by the HST. The microlensing source star is the blue star near the centre of the figure, which is partially blended with a much redder object (indicated by the arrow) displaced by $0.134''$. The direction of motion of the lens on the sky derived from the HST ($\theta_{\text{HST}} = -92^\circ$) and unconstrained parallax fit ($\theta_{\text{par}} = -100^\circ$) are both shown. **b**, The lens motion on the plane of the sky includes both the physical motion through space (proper motion) and an apparent small elliptical contribution due to the Earth's orbit around the Sun (parallax motion). The motion is plotted in equatorial coordinates in terms of right ascension, α , and declination, δ . At a distance of 200 pc, the parallax contribution has a diameter of $0.01''$. The overlaid grid shows the pixel size of the high resolution channel of the HST Advanced Camera for Surveys. Future measurements with this instrument may allow direct measurement of the lens parallax.

Table 1 LMC-5 HST image parameters

Star	V	$(V - R)$	$(V - I)$	μ (arcsec yr ⁻¹)	θ_{sky}^* (deg)
Source	21.016 ± 0.062	0.295 ± 0.091	0.677 ± 0.101		
Lens	22.666 ± 0.097	1.597 ± 0.124	3.175 ± 0.113	0.0214 ± 0.0007	-91.58 ± 0.95

* Direction of motion given in ecliptic coordinates.

the red object in the HST image is the lens, and consider how the microlensing system has evolved in time between peak magnification in 1993 and 1999, when the HST image was taken. The high magnification of this event suggests a very small impact parameter³, so at the peak of the lensing event, the lens and source were concentric to a very small fraction of a Planetary Camera pixel. The current separation of the source star and lens in the HST image then gives us the apparent proper motion of the lens relative to the source, μ_{rel} , and its direction of motion on the sky, θ_{sky} . The calibrated HST photometry⁷ and proper motion information are summarized in Table 1.

Some of the properties of the lens can also be determined from a microlensing parallax fit to the ground-based MACHO light-curve data. In a parallax fit, the effects of the Earth's motion around the Sun are included in addition to the normal parameters of a standard blend fit⁸. This type of fit to the ground-based light curve determines the transverse velocity projected to the solar position, $\tilde{v}$, the lens direction of proper motion θ_{par} and the event duration, $\hat{t}$. We performed two fits—an unconstrained parallax fit, and a constrained fit in which the lens flux and direction of motion are constrained to match the HST image. The actual variables used in the fit differ between the constrained and unconstrained fits; see Table 2. Errors are shown for the fit parameters, but not the derived parameters. We also show the results for a standard (non-parallax) blend fit.

The HST and light-curve data can be combined for a complete solution to the microlensing system. The lens mass may be expressed in terms of the observable variables as

$$m = \frac{c^2}{16G} \tilde{v} \hat{t}^2 \mu_{\text{rel}} \quad (1)$$

where G is the gravitational constant, and c is the speed of light⁹. This expression yields $m = 0.039 M_{\odot}$ (where $M_{\odot}$ is the solar mass) for the unconstrained fit, but, for accurate errors on the mass, we must use the constrained fit which uses a fit parameter proportional to m . The constrained fit yields $m = 0.036^{+0.009}_{-0.004} M_{\odot}$, which appears to indicate that the lens mass is sub-stellar at $(4-5)\sigma$. However, a direct calculation finds upper limits on the mass of $m \leq 0.069 M_{\odot}$ and $m \leq 0.097 M_{\odot}$ at the 2σ and 3σ confidence levels, respectively. Thus, a stellar-mass lens is allowed at the $(2-3)\sigma$ confidence level (depending on the assumed metallicity).

Furthermore, the relative proper motion (μ_{rel} , in units of arcsec yr⁻¹) is related to the relative parallax, π_{rel} (in units of arcsec), through the transverse velocity ($\tilde{v}$, in units of AU yr⁻¹) by

$$\tilde{v} \mu_{\text{rel}}^{-1} = \pi_{\text{rel}}^{-1} \approx \pi_1^{-1} \quad (2)$$

where the final approximation results from the assumption that the lens is much closer to the observer than the LMC source star. The lens parallax, π_1 (in units of arcsec) directly relates to the lens

distance d_l (in pc): $d_l = \pi_1^{-1}$. We find $d_l = 200^{+40}_{-30}$ pc, the absolute magnitude $M_V = 16.2^{+0.6}_{-0.5}$ and $v_t = \tilde{v}(1 - d_l/d_s) = 20^{+3}_{-4}$ km s⁻¹ (values for the constrained fit), where v_t is the velocity of the lens transverse to the line of sight, and d_s is the distance to the source star.

In the unconstrained parallax fit, we also have an additional parameter which is measured independently in the HST and light-curve analyses. This parameter, the lens direction of proper motion, θ_{par} , may be used to check the consistency of the HST and parallax data. Before a direct comparison with the HST value may be made, we must subtract from θ_{sky} the contribution of the parallax ellipse of radius (0.005") giving $\theta_{\text{HST}} = -89^{+1}_{-1}$ degrees. This is consistent with the parallax estimate of $\theta_{\text{par}} = -97^{+5}_{-6}$ degrees to within 1.6σ . Moreover, a comparison of the parallax and standard blend fits (see Table 2) shows similar lensed and unlensed flux fractions, but very different event durations. This is a consequence of the Earth's direction of motion at peak magnification, $\theta_{\text{Earth}} = -98^\circ$, so that the Earth's orbital velocity nearly cancels the relative transverse velocity between the lens and source.

Finally, a spectrum of the source-lens system was obtained with the European Southern Observatory Very Large Telescope (VLT). The present source-lens separation of 0.2" is unresolvable, and so the spectrum shown in Fig. 2 is a composite source-lens spectrum which becomes increasingly dominated by the lens flux at wavelengths greater than 7,000 Å and suggests that the lens is of spectral type M4-5V. The spectral type is also consistent with the HST colours¹⁰. The relation between mass and spectral type depends somewhat on the (unknown) metallicity of the lens, but a spectral type of M4-5 implies $m \approx (0.095 - 0.13) M_{\odot}$ (ref. 11), inconsistent by at least 3σ with the central value of the parallax fit. Using an empirical relation between colour and absolute magnitude for observed M dwarfs¹², $M_V = 2.89 + 3.37(V - I)$, with a colour-dependent dispersion¹³ of about 0.4 at $(V - I) \approx 3$, we obtain $M_V = 13.61 \pm 0.55$, and $d_l = 650 \pm 190$ pc. Thus, the photometric lens distance is also inconsistent at more than 2σ with the parallax fit distance. We note, however, that these two inconsistencies are not independent, because higher-mass objects are brighter and more distant at a given apparent magnitude.

A heliocentric radial velocity was computed using the potassium and sodium lines of the lens, and equals $v_r = 49 \pm 10$ km s⁻¹. Along with the proper motion and distance from the HST and parallax fit, this allows us to solve for the space motion of the lens. At a distance of 200 pc, the velocity of the lens (relative to the local standard of rest) is 27 km s⁻¹, -43 km s⁻¹ and -4 km s⁻¹ for the respective velocity components towards the Galactic Centre, in the direction of Galactic rotation and up out of the Galactic plane. An orbit calculated with this space motion and a standard Galactic potential suggests that the lens belongs to the disk, not the halo. Furthermore, at 200 pc the lensing probability is also many times higher for a lens from a disk density distribution than from a halo distribution.

Table 2 LMC-5 light-curve blend fit parameters

Method	t_0^*	$f_R^\dagger$	$f_V^\dagger$	$\tilde{f}_R^\dagger$	$\tilde{f}_V^\dagger$	i (d)	$\tilde{v}$ (km s ⁻¹)	m ($M_{\odot}$)	θ_{PAR} (deg)
Unconstrained	1.3	$29.3^{+4.6}_{-4.4}$	$29.8^{+4.8}_{-4.7}$	$-12.7^{+4.5}_{-4.5}$	$5.4^{+4.7}_{-4.8}$	$45.08^{+0.38}_{-0.36}$	$-18.42^{+1.83}_{-1.91}$	0.039	$-97.15^{+4.82}_{-6.33}$
Constrained	10.8	$23.0^{+2.2}_{-2.2}$	$23.3^{+2.3}_{-2.3}$	$-6.43^{+2.2}_{-2.2}$	$11.9^{+2.3}_{-2.3}$	41.46	-20.12	$0.036^{+0.009}_{-0.004}$	$-91.82^{+2.04}_{-2.28}$
Standard	24.0	$28.9^{+3.1}_{-2.8}$	$29.6^{+3.3}_{-3.0}$	$-12.5^{+2.3}_{-3.0}$	$5.6^{+3.0}_{-3.2}$	$65.94^{+5.70}_{-5.10}$			

* The time of peak magnification t_0 is given in MJD - 49000.

† The parameters f and $\tilde{f}$ give the lensed and unlensed flux in arbitrary units in the MACHO red (R) and blue (V) bands. The lensed flux is the flux of the source star, while the unlensed flux is the flux of the lens plus any unresolved neighbouring stars in the ground-based MACHO images. Unresolved, but unblended, neighbouring stars are included as part of the fit 'sky' background. If the mean brightness of these more-distant neighbours exceeds that of the close neighbours, then the best-fit unlensed flux will be negative.

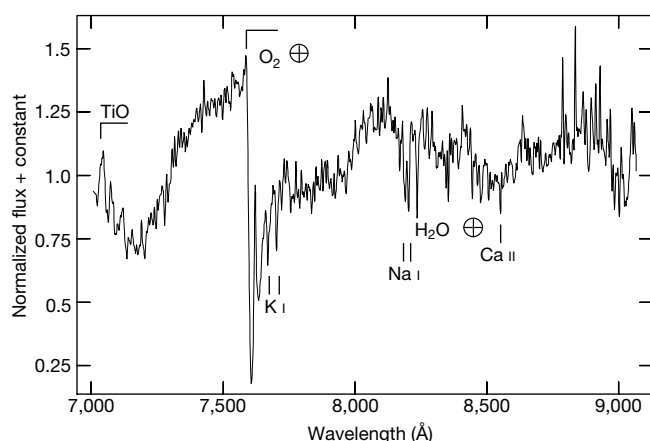


Figure 2 Spectrum of LMC-5. We show a composite VLT spectrum (using the FORS2 spectrograph) of the LMC-5 source-lens system from four 1,500-s exposures on 2 February 2001. The potassium, sodium and titanium oxide features from the lens are marked. The calcium lines are a blend from both the lens and the LMC source star (spectral type F). The presence of K I, Na I, the absence of Cs I + Rb I and the TiO band at 7,100 Å with corresponding absence of the VO band at 7,450 Å lead us to conclude that the lens is of spectral type M4-5V. The spectrum has been put on a relative flux scale, and smoothed to a resolution of about 3 Å.

This extended analysis of the LMC-5 microlensing system was motivated largely by the consistency of the lens direction of motion in the parallax fit and HST image. But further analysis of this system has led to some inconsistencies between results obtained with the HST proper motion and parallax fit, and results obtained with the HST photometry and spectra. Most notably, the parallax fit mass is consistent only at 3σ with the lower-limit mass allowed by the spectral type. Also, the parallax lens distance is consistent only at 2.3σ with the photometric distance. The inconsistencies may have arisen from difficulties in the parallax fit, as parallax effects are difficult to measure for such short events without much better sampling and photometry.

The conflict between the parallax and photometric solutions may be resolved in the future by using the HST Advanced Camera for Surveys, expected to become available in 2002. In the high-resolution channel, this instrument is expected to have a pixel size of 0.027", a factor of two better resolution than WFPC2 and with a critically sampled point spread function. Two epochs of data from this instrument would allow us to both confirm the proper motion of the lens and directly measure the parallax motion. □

Received 16 August; accepted 19 October 2001.

- Hogan, C. J. Gravitational lensing by cold dark matter catastrophes. *Astrophys. J.* **527**, 42–45 (1999).
- Tytler, D., O'Meara, J. M., Suzuki, N. & Lubin, D. Deuterium and the baryonic density of the universe. *Phys. Rev.* **333**, 409–432 (2000).
- Alcock, C. *et al.* The MACHO Project: Microlensing results from 5.7 years of Large Magellanic Cloud observations. *Astrophys. J.* **542**, 281–307 (2000).
- Alcock, C. *et al.* The MACHO Project Hubble Space Telescope follow-up: Preliminary results on the location of the Large Magellanic Cloud microlensing source stars. *Astrophys. J.* **552**, 582–590 (2000).
- Alcock, C. *et al.* The MACHO Project Large Magellanic Cloud microlensing results from the first two years and the nature of the Galactic dark halo. *Astrophys. J.* **486**, 697–726 (1997).
- Gould, A., Bahcall, J. N. & Flynn, C. M dwarfs from Hubble Space Telescope star counts. III. The Groth Strip. *Astrophys. J.* **482**, 913–918 (1997).
- Holtzman, J. A. *et al.* The photometric performance and calibration of WFPC2. *Proc. Astron. Soc. Pacif.* **107**, 1065–1093 (1995).
- Alcock, C. *et al.* First observation of parallax in a gravitational microlensing event. *Astrophys. J.* **454**, L125–L128 (1995).
- Gould, A. Extending the MACHO search to $\sim 10^6 M_\odot$. *Astrophys. J.* **392**, 422–451 (1992).
- Bessell, M. S. The late M dwarfs. *Astrophys. J.* **101**, 662–676 (1991).
- Cassisi, S., Castellani, V., Ciarcia, P., Pionto, G. & Zoccali, M. Galactic globular clusters as a test for very-low-mass stellar models. *Mon. Not. R. Astron. Soc.* **315**, 679–688 (2000).
- Reid, N. Unresolved binaries and the stellar luminosity function. *Astron. J.* **102**, 1428–1438 (1991).
- Zheng, Z., Flynn, C., Gould, A., Bahcall, J. N. & Salim, S. M dwarfs from Hubble Space Telescope star counts. IV. *Astrophys. J.* **555**, 393–404 (2001).

Acknowledgements

We thank the ESO director for discretionary time on the VLT. This work was supported by NASA and the Space Telescope Science Institute, which is operated by the Association of Universities for Research in Astronomy. This work was performed under the auspices of the US Department of Energy by the University of California, Lawrence Livermore National Laboratory. Work performed by the Center for Particle Astrophysics personnel is supported in part by the Office of Science and Technology Centers of the NSF. C.W.S. thanks the Packard foundation for support. W.J.S. is supported by a PPARC advanced fellowship. C.A.N. is supported in part by an NPSC Graduate Fellowship. T.V. and K.G. were supported in part by the US DOE. D.M. is supported by Fondecyt.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to C.A.N. (e-mail: cnelson@igpp.ucllnl.org).

Electrical control of spin coherence in semiconductor nanostructures

G. Salis^{*}, Y. Kato^{*}, K. Ensslin^{*†}, D. C. Driscoll^{*}, A. C. Gossard^{*} & D. D. Awschalom^{*}

^{*} Center for Spintronics and Quantum Computing, University of California, Santa Barbara, California 93106, USA

[†] Solid State Physics, ETH Zürich, 8093 Zürich, Switzerland

The processing of quantum information based on the electron spin degree of freedom^{1,2} requires fast and coherent manipulation of local spins. One approach is to provide spatially selective tuning of the spin splitting—which depends on the g -factor—by using magnetic fields³, but this requires their precise control at reduced length scales. Alternative proposals employ electrical gating⁴ and spin engineering in semiconductor heterostructures involving materials with different g -factors. Here we show that spin coherence can be controlled in a specially designed $\text{Al}_x\text{Ga}_{1-x}\text{As}$ quantum well in which the Al concentration x is gradually varied across the structure. Application of an electric field leads to a displacement of the electron wavefunction within the quantum well, and because the electron g -factor varies strongly with x , the spin splitting is therefore also changed. Using time-resolved optical techniques, we demonstrate gate-voltage-mediated control of coherent spin precession over a 13-GHz frequency range in a fixed magnetic field of 6 T, including complete suppression of precession, reversal of the sign of g , and operation up to room temperature.

The spin splitting of electrons confined in a quantum well depends on the g -factors of both the well and the barrier material, owing to penetration of the wavefunction into the energetically forbidden barrier^{4,5}. It has been shown theoretically⁶ and experimentally⁷ that this splitting can be changed in a GaAs/AlGaAs quantum well by pushing the tail of the wavefunction into the barrier, using a bias. But the shifts measured to date are relatively small, because of the low probability density in the barrier. Here we substantially increase the tunability by using a heterostructure with a potential profile that allows spatial displacement of the full, unperturbed wavefunction. This profile is achieved by gradually varying the Al concentration x of $\text{Al}_x\text{Ga}_{1-x}\text{As}$ layers between $\text{Al}_{0.4}\text{Ga}_{0.6}\text{As}$ barriers, thereby realizing a parabolic confinement potential in the conduction band. When an electric field is applied across such a parabolic quantum well, the potential minimum is spatially displaced, while its shape is preserved. This leads to a translation of an unchanged electron wavefunction⁸ and the

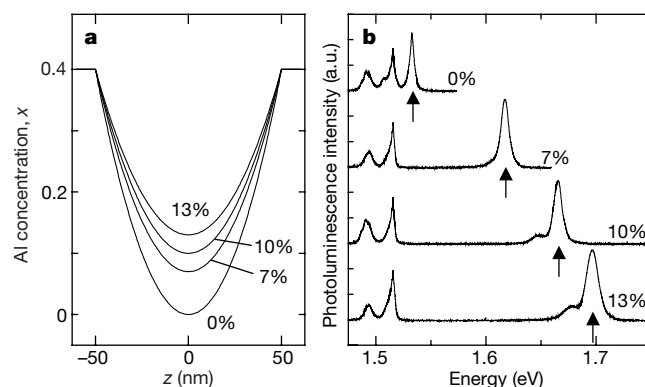


Figure 1 Sample design. **a**, The Al concentration of the four different samples is varied parabolically from 0%, 7%, 10% and 13% in the centre to 40% at the barriers.

b, Photoluminescence of the four samples. The quantum-well peaks (marked by vertical

arrows) shift with increasing Al concentration to higher energies, corresponding to the change of the bandgap. Remaining peaks arise from the GaAs substrate luminescence. a.u., arbitrary units.

sampling of different Al concentrations as a function of the applied electric field. Exploiting the fact that the electron g -factor varies strongly with x and changes sign (in bulk $\text{Al}_x\text{Ga}_{1-x}\text{As}$, $g = -0.44$ for $x = 0$ and $g = 0.40$ for $x = 0.3$) (ref. 9), we show that a low-voltage electrical bias can efficiently tune and even invert the spin splitting in these engineered structures. In particular, this ability to achieve a zero g -factor and quench electron spin precession provides opportunities for disentangling the spin and orbital degrees of freedom in nanostructures.

Four different samples were grown by molecular beam epitaxy¹⁰ on semi-insulating GaAs (100) with $x = 0\%$, 7% , 10% and 13% in the centre of the well (Fig. 1a). In each sample, the Al concentration was increased quadratically towards the barriers, where it reached $x = 40\%$. The width of each well was 100 nm, and the quantum wells were nominally undoped. A silicon-doped layer located 1.1 μm below the surface served as a back gate, and was electrically isolated from the quantum well region by 500 nm of low-temperature-grown GaAs (ref. 11). The back gate was electrically contacted using annealed AuGe/Ni, and a thin transparent front gate consisting of 20 Å titanium and 50 Å gold was evaporated onto the surface of the sample. Applying a voltage U_g between the back and front gates allowed an electric field to be established across the structure. A positive value of U_g corresponds to a positive voltage at the back gate with respect to the front gate.

Figure 1b shows photoluminescence measurements of the as-grown samples at $T = 5$ K. The carriers were excited with a He-Ne laser (1.962 eV) of intensity $\sim 0.1 \text{ W cm}^{-2}$. Peaks related to the quantum well emission are located at 1.533 eV, 1.617 eV, 1.665 eV and 1.696 eV for the four samples, reflecting the increase in the bandgap with increasing Al concentration.

Spin precession was monitored with time-resolved Kerr rotation (TRKR)¹² using a Ti:sapphire laser with 100-fs pulses and a repetition rate of 76 MHz. Circularly polarized pump pulses (0.64 mW focused to an $\sim 70 \mu\text{m}$ spot) tuned to the absorption edge of the quantum well inject spin-polarized electron-hole pairs. Linearly polarized probe pulses ($\sim 40 \mu\text{W}$) arrive at time Δt later, and are reflected from the sample. Owing to the Kerr effect, the polarization of the reflected light is rotated by an angle θ proportional to the spin-polarization of the quantum-well electrons along the beam direction. By measuring θ as a function of Δt in the presence of a magnetic field B applied in the quantum-well plane, we measure the coherent spin precession—whose time evolution can be described as $A \exp(-\Delta t/T_2^*) \cos(\Omega \Delta t)$, where A is the amplitude, T_2^* the inhomogeneous transverse electron spin lifetime and Ω the angular precession frequency. We note that the observed spin precession is of conduction-band electrons, and not of excitons¹³ or holes.

Figure 2a shows TRKR data for the $x = 7\%$ sample at different

values of U_g , taken at $T = 5$ K and a constant magnetic field $B = 6$ T, with the pump and probe energy fixed at 1.644 eV. As the electron wavefunction is pushed toward the back gate (Fig. 2b), the spin dynamics change markedly. Initially, Ω decreases with increasing U_g until the optically injected electron spin precession is completely suppressed within the spin lifetime of ~ 200 ps. At higher gate voltages, the spins precess again and Ω increases with U_g . A fit to the Kerr rotation data yields Ω and hence the electron g -factor using $g = \hbar \Omega / B \mu_B$, where μ_B is the Bohr magneton. Figure 3a shows g as a function of U_g between -4 V and 3 V for all four samples at $T = 5$ K. The laser energy is tuned to 1.563 eV, 1.644 eV, 1.676 eV and 1.702 eV for the samples with $x = 0\%$, 7% , 10% and 13% , respectively. As expected, varying x leads to a change in g . Although the sign of g cannot be obtained directly from the experiment, the assumption of a monotonic increase of g with x suggests that g is positive for all gate voltages in the 10% and 13% sample, while g remains negative for the 0% sample. The minima of g are shifted in voltage with respect to zero bias owing to the built-in potential U_0 . As noted previously (Fig. 2), the $x = 7\%$ sample displays a zero crossing of g at $U_g = 2$ V. The accessibility of $g = 0$ with this sample

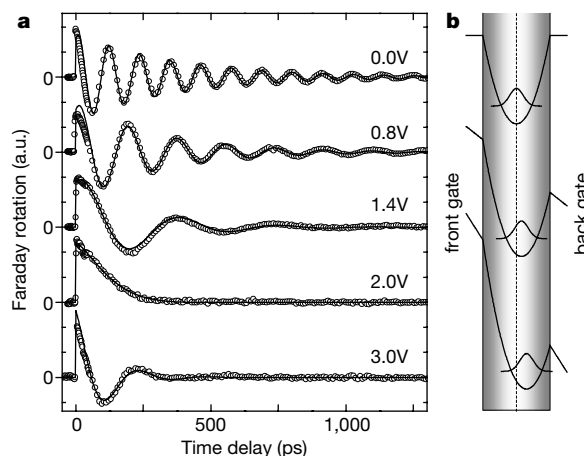


Figure 2 Voltage-controlled spin coherence. **a**, Time-resolved Kerr rotation measurements of the electron spin precession in the quantum well at different gate voltages U_g with Al concentration of 7% at 5 K and $B = 6$ T. As a positive voltage U_g is applied between back and front gate, the electron wavefunction is displaced towards the back gate into regions with more Al concentration (diagram in **b**), leading to an increase of g . At $U_g = 2$ V, no precession is observed, corresponding to $g = 0$. Circles are data points, and the solid lines are fits to the data as explained in the text.

allows continuous tunability through zero, including quenching and reversal of the spin precession frequency despite the applied magnetic field. The g -factor can be varied by ~ 0.16 ($-0.11 < g < +0.05$), corresponding to a change in spin precession frequency of 13 GHz. In addition, reversal of the g -factor through changes in bias can result in the inversion of Zeeman-split spin populations.

In the $x = 7\%$ sample, the g -factor remains nearly constant for $-3 \text{ V} < U_g < 0 \text{ V}$, and only starts to increase for $U_g > 0 \text{ V}$ and $U_g < -3 \text{ V}$. This can be understood by measuring the gate-voltage dependence of the photoluminescence. Figure 3b shows the photoluminescence intensity and peak position as a function of U_g at $T = 5 \text{ K}$, where pulsed excitation is used with an average intensity of $\sim 1 \text{ W cm}^{-2}$ and energy of 1.676 eV. The decrease of photoluminescence intensity away from U_0 arises from reduced wavefunction overlap between photoexcited electrons and holes with increasing electric field, leading to dissociation of the exciton. Calculations yield a full-width at half-maximum of the electronic probability density of $\sim 16 \text{ nm}$, and a displacement in the z -direction (growth direction) of $\sim 5 \text{ nm V}^{-1}$ for electrons and $\sim 7.5 \text{ nm V}^{-1}$ for holes. These estimates qualitatively explain the gate voltages for which photoluminescence can be observed. This range corresponds roughly to the region of constant g , suggesting that the excitonic Coulomb energy keeps electron and holes in the region of minimal Al content until the electric field dissociates the exciton and consequently displaces the electron wavefunction into regions with higher Al concentration. In addition, the photoluminescence energy decreases with U_g away from $U_g \approx U_0$, in agreement with the quantum-confined Stark effect¹⁴. The observed shift is $\sim 9 \text{ meV V}^{-2}$ compared to a theoretical value of 5.2 meV V^{-2} using conduction

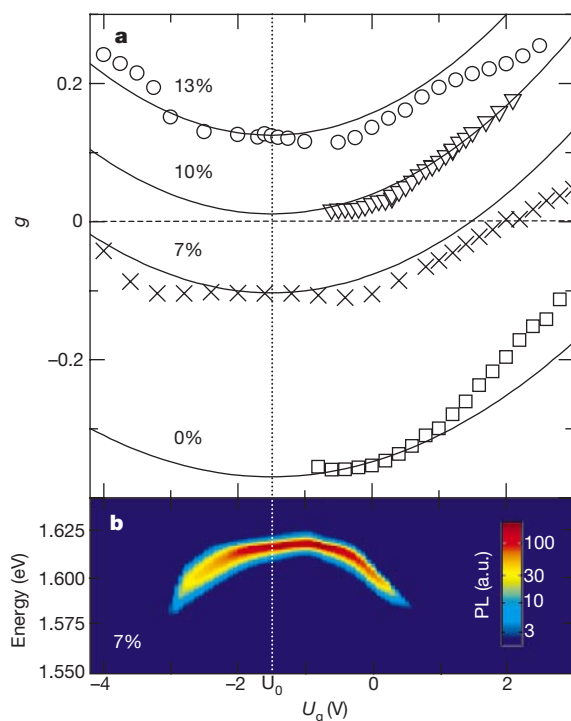


Figure 3 Electrical tuning of electron spin and charge dynamics. **a**, g -factors extracted from time-resolved Kerr rotation measurements as a function of U_g (symbols) for the four samples at $B = 6 \text{ T}$ and $T = 5 \text{ K}$. Solid lines are fits to the data using a weighted average over the bulk g -factor. The two samples with 7% and 13% Al concentration were grown and processed in a separate run, and can be operated at voltages down to -4 V , whereas the 0% and 10% samples display large leakage currents for $U_g < -0.5 \text{ V}$. **b**, Photoluminescence (PL) intensity in a logarithmic colour plot as a function of gate voltage for the $x = 7\%$ sample.

and valence band offsets from the literature¹⁵ and neglecting changes in the exciton binding energy, which are of the order of 6 meV (ref. 16).

In order to quantitatively interpret the data in Fig. 3a we use a simple model that averages the g -factor over the well width weighted by the electron wavefunction. Given that the density of optically created electrons and holes is of the order of 10^{10} cm^{-2} , only the lowest subband is occupied, and self-consistent effects leading to a screening of the parabolic potential can be neglected. We use the ground-state envelope wavefunction $\psi(z) = l^{-1/2} \pi^{-1/4} \exp(-z^2/2l^2)$ of a parabolic potential $\phi(z) = m\omega^2 z^2/2$, where $l = (\hbar/m\omega)^{1/2}$ is the characteristic length, ω the angular frequency and m the effective electron mass. By averaging over the electron probability density $|\psi(z)|^2$, we obtain $g = \int g(x(z))|\psi(z)|^2 dz$. The function $g(x)$ describes the g -factor of bulk $\text{Al}_x\text{Ga}_{1-x}\text{As}$, and $x(z)$ is the Al concentration in the layer at position z . Applying a bias U_g between front and back gate displaces the wavefunction by $e(U_g - U_0)/\omega^2 md$, where $d = 1.1 \mu\text{m}$ is the distance between front and back gate and e the electron charge. For $g(x)$ we use an extrapolated linear dependence through $g(0) = -0.44$ and $g(0.12) = +0.02$ (ref. 9). A constant offset Δg_c is added owing to confinement effects, which are not inherently included in our simple model. By taking $\Delta g_c = 0.05$ and $U_0 = -1.5 \text{ V}$, the data for all four samples in Fig. 3a can be simultaneously fitted (solid lines) very well.

Figure 4a shows the temperature and gate-voltage dependence of g , demonstrating that the tunability of the electron g -factor persists to room temperature. Raising the temperature leads to an increase of g , which has also been reported for bulk GaAs (ref. 17). The tunability of g decreases with higher T , and with increased laser intensity (not shown). In both cases, the leakage current between front and back gate increases, which might lead to a reduction of the effective voltage drop across the quantum well. The flat region in g around U_0 broadens at higher temperatures, consistent with its attribution to excitons which dissociate more readily at elevated temperatures.

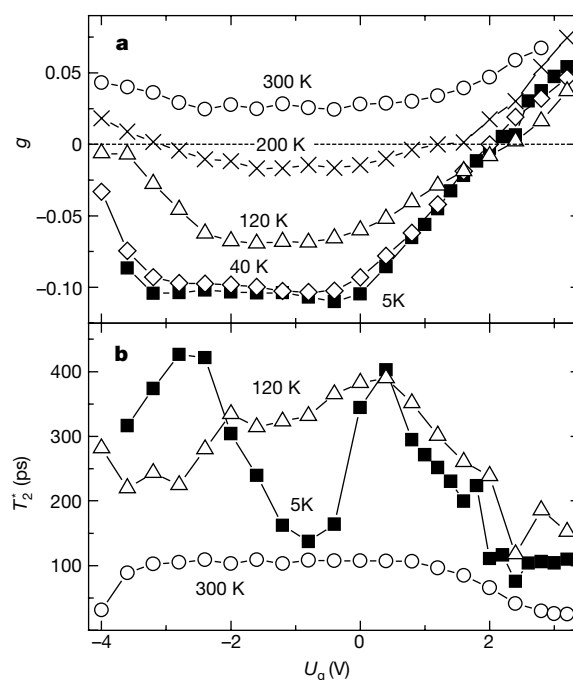


Figure 4 Tunability of electron spin coherence at different temperatures. **a**, The electron g -factor increases with temperature, and is positive for all gate voltages at temperatures above $\sim 240 \text{ K}$. **b**, The spin lifetime T_2 is limited by charge recombination at 5 K and $U_g = -1 \text{ V}$.

For coherent manipulation of spin states, it is desirable for the spin coherence lifetime to be much longer than the time it takes to rotate a spin by π , which is ~ 40 ps for $g = 0.15$ and $B = 6$ T. A lower bound for the coherence time is the measured T_2^* , which is shown in Fig. 4b as a function of U_g for $T = 5$ K, 120 K and 300 K. Clearly, T_2^* exceeds 40 ps throughout the entire temperature and voltage range, thus making rapid operations possible within the dephasing time. At $T = 5$ K, T_2^* substantially decreases from ~ 450 ps to 140 ps near $U_g = -1$ V. This can be attributed to an enhanced charge recombination rate, because time-resolved photoluminescence measurements reveal a marked decrease of the radiative lifetime from several nanoseconds to ~ 250 ps in this region. At higher T , this dip successively disappears and evolves into a maximum at $T > 120$ K. At $T = 120$ K, the radiative lifetime is ~ 1 ns, much longer than the measured T_2^* . Another possible explanation is the formation of excitons, which may limit the spin lifetime at low temperature¹⁸.

This ability to tune the electron g -factor is in principle applicable to local spins by lateral patterning of the gate electrodes¹, and constitutes a potential basis for single-qubit operations in quantum information processing schemes. With longer spin relaxation times expected in quantum dots¹⁹ coherent manipulation of spins with patterned high-frequency electronic gates may be possible. Furthermore, the large tunability and quenching of the electronic spin splitting offers the potential for insights into other phenomena, such as ferromagnetic quantum Hall states²⁰ or the dynamics of electrically inverted spin populations through non-adiabatic gating. □

Received 29 August; accepted 10 October 2001.

1. Loss, D. & DiVincenzo, D. P. Quantum computation with quantum dots. *Phys. Rev. A* **57**, 120–126 (1998).
2. Kane, B. E. A silicon-based nuclear spin quantum computer. *Nature* **393**, 133–137 (1998).
3. DiVincenzo, D. P. *et al.* Quantum computing and single qubit measurements using the spin filter effect. *J. Appl. Phys.* **85**, 4785–4787 (1999).
4. Snelling, M. J. *et al.* Magnetic g -factor of electrons in GaAs/Al_xGa_{1-x}As quantum wells. *Phys. Rev. B* **44**, 11345–11352 (1991).
5. Kowalski, B. *et al.* Conduction-band spin splitting of type-I Ga_xIn_{1-x}As/InP quantum wells. *Phys. Rev. B* **49**, 14786–14789 (1994).
6. Ivchenko, E. L., Kiselev, A. A. & Willander, M. Electronic g -factor in biased quantum wells. *Solid State Commun.* **102**, 375–378 (1997).
7. Jiang, H. W. & Yablonovitch, E. Gate-controlled electron spin resonance in GaAs/Al_xGa_{1-x}As heterostructures. *Phys. Rev. B* **64**, R41307–R41310 (2001).
8. Salis, G. *et al.* Wave function spectroscopy in quantum wells with tunable electron density. *Phys. Rev. Lett.* **79**, 5106–5109 (1997).
9. Weisbuch, C. & Hermann, C. Optical detection of conduction-electron spin resonance in GaAs, Ga_{1-x}In_xAs, and Ga_{1-x}Al_xAs. *Phys. Rev. B* **15**, 816–822 (1977).
10. Gossard, A. C. Growth of microstructures by molecular beam epitaxy. *IEEE J. Quant. Electr.* **22**, 1649–1655 (1986).
11. Maranowski, K. D., Ibbetson, J. P., Campman, K. L. & Gossard, A. C. Interface between low-temperature grown GaAs and undoped GaAs as a conduction barrier for back gates. *Appl. Phys. Lett.* **66**, 3459–3461 (1995).
12. Kikkawa, J. M., Smorchkova, I. P., Samarth, N. & Awschalom, D. D. Room-temperature spin memory in two-dimensional electron gases. *Science* **277**, 1284–1287 (1997).
13. Amand, T. *et al.* Spin quantum beats of 2D excitons. *Phys. Rev. Lett.* **78**, 1355–1358 (1997).
14. Miller, D. A. B. *et al.* Band-edge electroabsorption in quantum well structures: the quantum-confined Stark effect. *Phys. Rev. Lett.* **53**, 2173–2176 (1984).
15. Miller, R. C., Gossard, A. C. & Kleinmann, D. A. Band offsets from two special GaAs-Al_xGa_{1-x}As quantum-well structures. *Phys. Rev. B* **32**, 5443–5446 (1985).
16. Chu-liang, Y. & Qing, Y. Sublevels and excitons in GaAs-Al_xGa_{1-x}As parabolic-quantum-well structures. *Phys. Rev. B* **37**, 1364–1367 (1988).
17. Oestreich, M. & Rühle, W. W. Temperature dependence of the electron Lande g -factor in GaAs. *Phys. Rev. Lett.* **74**, 2315–2318 (1995).
18. Bir, G. L., Aronov, A. G. & Pikus, G. E. Spin relaxation of electrons scattered by holes. *Sov. Phys. JETP* **42**, 705–712 (1976).
19. Paillard, M. *et al.* Spin relaxation quenching in semiconductor quantum dots. *Phys. Rev. Lett.* **86**, 1634–1637 (2001).
20. De Poortere, E. P., Tutuc, E., Papadakis, S. J. & Shayegan, M. Resistance spikes at transitions between quantum Hall ferromagnets. *Science* **290**, 1546–1549 (2000).

Acknowledgements

We thank J. Kotthaus, M. E. Flatté, I. Meinel and R. K. Kawakami for discussions. This work was supported by DARPA, ONR and NSF.

Correspondence and requests for materials should be addressed to D.D.A. (e-mail: awsch@physics.ucsb.edu).

Breakdown of intermediate-range order in liquid GeSe₂ at high pressure

Wilson A. Crichton*, Mohamed Mezouar*, Tor Grande†, Svein Stølen‡ & Andrzej Grzechnik§

* European Synchrotron Radiation Facility, BP 220, F-38043 Grenoble, France

† Department of Chemistry, Norwegian University of Science and Technology, N-7034 Trondheim, Norway

‡ Department of Chemistry, University of Oslo, Postbox 1033 Blindern, N-0315 Oslo, Norway

§ Max-Planck-Institut für Festkörperforschung, Heisenbergstr. 1, D-70569 Stuttgart, Germany

Studies of liquids with tetrahedral coordination, particularly during compression or quenching, have indicated the existence of distinct phases^{1–3} in the liquid state, distinguishable by density and local structure. In systems that exhibit critical phenomena in the supercooled state, anomalous behaviour of the compressibility is also anticipated above the critical point, as revealed by simulations of water⁴. Liquid GeSe₂ is a potentially attractive system for studying both types of phenomena, given its two-dimensional tetrahedral structure and anomalous physical properties (including a density minimum near its melting point). Here we report *in situ* X-ray diffraction measurements of solid and liquid GeSe₂ at high temperature and high pressure, revealing that the structure of the liquid is sensitive to pressure and that anomalous compressibility is expected. During compression of liquid GeSe₂, the connectivity of the liquid changes from two- to three-dimensional, leading to a breakdown of the intermediate-range order. The gradual change in structure above the melting line may develop to a first-order liquid–liquid transition in the supercooled regime.

A first-order transition between two liquids of different densities is consistent with experimental findings and molecular dynamics simulations for a variety of substances, including water^{4,5}, silica⁶ and carbon⁷. Indirect evidence for liquid–liquid transitions for some elements has also been found in studies of I, S and Se (refs 1–3), elements that show molecular structure and low density at ambient pressure. Moreover, the coexistence of two phases that are chemically identical but have different density and local structure has been observed in quenched Y₂O₃–Al₂O₃ melts⁸. All these reports have encouraged the search for polymorphic phase transitions in liquids and glasses. So far, there have been no reports of such phenomena in GeSe₂, in spite of its anomalous behaviour. For example, three amorphous GeSe₂ phases with completely different diffraction patterns were obtained by compression of GeSe₂ glass to different pressures in the vicinity of the glass transition⁹. In a separate report, an abrupt change in the ambient conductivity of GeSe₂ glass was observed at 7 GPa during compression¹⁰. In contrast, compression of crystalline GeSe₂ resulted in pressure-induced amorphization at 14 GPa (ref. 11), while no indication of a transition near 7 GPa was observed. One unusual character of liquid GeSe₂ is its negative expansivity¹². By extrapolation of the volume of GeSe₂ glass above the glass transition and the volume of liquid GeSe₂, a density minimum is inferred just below the ambient melting point. The negative expansivity of the liquid can be understood in terms of increasing chemical disorder with increasing temperature¹³. This is also in line with the negative excess volume of liquid GeSe₂ relative to the volume of pure Ge and Se (ref. 12).

Here we report the pressure–temperature (P – T) phase diagram of GeSe₂, and *in situ* high-temperature and high-pressure diffraction data for liquid GeSe₂. The P – T phase diagram for GeSe₂ is shown in Fig. 1. We observed only two crystalline polymorphs of GeSe₂: the two-dimensional (2D) monoclinic GeSe₂, stable at

ambient pressure¹⁴, and tetragonal GeSe₂ (three-dimensional; 3D), stable at high pressure¹⁵. The 2D polymorph consists of edge-sharing and corner-sharing GeSe₄ tetrahedra, while only corner-sharing tetrahedra are present in tetragonal (3D) GeSe₂, where 2D and 3D indicate the dimensionality of the tetrahedral network. Below 1 GPa, monoclinic GeSe₂ transforms to tetragonal GeSe₂ (ref. 9), as shown in Fig. 1. The Ge–Se bond distance of about 2.36 Å, equal to the Pauling radii for Ge and Se, is almost equal in monoclinic and tetragonal GeSe₂ (ref. 15). The difference in density between the two polymorphs¹⁵ (9.6%) is therefore entirely due to the change in connectivity between the GeSe₄ tetrahedra. A positive slope of the *P*–*T* line for the monoclinic–tetragonal phase transition is anticipated, owing to the higher density of tetragonal GeSe₂.

Based on thermodynamic data, the initial slope (*dT/dP*) of the melting line for GeSe₂ (2D) is +90 K GPa^{−1} (see Fig. 1). Despite the uncertainty in our determinations of pressure and temperature, we anticipate a melting-point maximum below 0.5 GPa for monoclinic GeSe₂ from the determination of the melting line above 0.5 GPa. We estimate that the triple point, where monoclinic and tetragonal GeSe₂ coexist with the liquid, occurs at 0.9 ± 0.1 GPa and 950 ± 75 K. The melting line of tetragonal GeSe₂ (3D) has a positive slope, reflecting a positive volume change upon melting. Compression of tetragonal GeSe₂ is manifest in a Ge–Se bond length that is insensitive to pressure, and the fact that the compressibility is governed by decreasing Ge–Se–Ge bond angle¹⁶.

The structure of liquid GeSe₂ at ambient pressure mimics the short-range and intermediate-range order of monoclinic GeSe₂ (refs 13, 17). The principal structural entity is the GeSe₄ tetrahedron: tetrahedra are linked by both edge and corner sharing, giving the liquid a 2D character. The edge-sharing tetrahedra represent a fundamental difference between GeSe₂ and other better known tetrahedral substances such as water and silica. Another important difference is that adding Ge or Se easily chemically modifies GeSe₂. Chemical disorder or Ge–Ge and Se–Se bonds are expected to occur in liquid GeSe₂ at elevated temperature, owing to the relatively low endothermic dissociation enthalpy and high positive dissociation entropy of GeSe₂ (refs 18, 19). The chemical disorder is also expected in such solutions owing to concentration fluctuations at elevated temperatures. Evidence for increasing chemical disorder in molten GeSe₂ with increasing temperature has also been provided

by neutron diffraction using isotope-substituted samples¹³. Chemical disorder at high temperatures is also supported by molecular dynamics simulations of liquid GeSe₂ (ref. 20).

The structure factors, *S*(*Q*), where *Q* is the wavenumber, of liquid GeSe₂ are shown in Fig. 2 for increasing pressure (0.5–4.1 GPa) under nearly isothermal conditions at around 1,120 K. Additional scans also shown in Fig. 2, at 5.1 and 1.1 GPa and 1,223 K, demonstrated the reversibility of the pressure effect. The structure factors below 2.5 GPa show features widely associated with GeSe₂ glasses and liquids, in that they have the first sharp diffraction peak (FSDP) at $\sim 0.9 \text{ \AA}^{-1}$. The reduction in the FSDP is accompanied by a shift to higher *Q* with increasing pressure. At higher *Q* in Fig. 2, the peaks associated with the Ge–Se and Se–Se, Ge–Ge correlations are observed at $\sim 2.1 \text{ \AA}^{-1}$ and $\sim 3.6 \text{ \AA}^{-1}$ (ref. 17). A closer examination of the peak positions and widths in *S*(*Q*) reveals a shift in position of the second peak accompanied by peak broadening and a strong damping of the third peak relative to the second.

The most important observation is the clear reduction in the amplitude of the FSDP with increasing pressure; the FSDP almost disappears at pressures above ~ 2.5 GPa. Reduction and shift in the FSDP has also been observed with increasing temperature by neutron diffraction of liquid GeSe₂ (ref. 17). The FSDP is a manifestation of intermediate-range order on length scales larger than those typical of nearest-neighbour distances^{13,17,20,21}. In liquid GeSe₂, the FSDP arises predominantly from the Ge–Ge correlations owing to the spatial periodicity of the GeSe₄ tetrahedra^{13,17}, but the structural origin of the FSDP is still a matter of discussion²¹. The disappearance of the FSDP shows that the intermediate structure is sensitive to pressure.

A change in the connectivity between the GeSe₄ tetrahedra is anticipated from the crystal structure of tetragonal GeSe₂. The change in the structure of GeSe₂ with increasing temperature mimics that observed in the liquid as Ge is added to GeSe₂ to form GeSe (ref. 17). The pressure dependence of *S*(*Q*) that we observe calls for a modified structural model, including the conversion to only edge-sharing tetrahedra and, further, a breakdown of the intermediate order. We find that the intensity of the second peak relative to that of the third peak increases with pressure, a change that was not observed with increasing temperature¹⁷.

In Fig. 3 we show the *G*(*r*) curves of the liquid patterns, obtained by Fourier transform of the *S*(*Q*) relations (the *G*(*r*) are a function

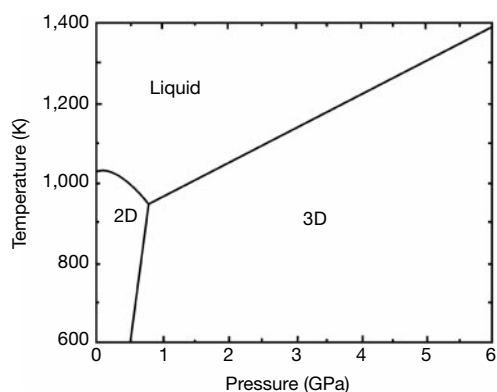


Figure 1 The *T*–*P* phase diagram of GeSe₂ up to 6 GPa. At ambient temperature, monoclinic GeSe₂ (2D) melts at 1,025 K (ref. 27). The initial slope of the melting line for 2D GeSe₂ is 90 K GPa^{−1} calculated by the Clausius–Clapeyron equation ($dT/dP = \Delta S/\Delta V$). The calculation used the estimated molar volume of fusion ($2.2 \text{ cm}^3 \text{ mol}^{-1}$) from the density data of liquid and 2D GeSe₂ (refs 12, 28) and the enthalpy of fusion of 2D GeSe₂ ($23.7 \text{ J K}^{-1} \text{ mol}^{-1}$; ref. 27). The transition from monoclinic (2D) to tetragonal (3D) GeSe₂ occurs below 1 GPa (ref. 9), and a positive *dT/dP* dependence for the transition is estimated on the basis of the higher density of 3D GeSe₂.

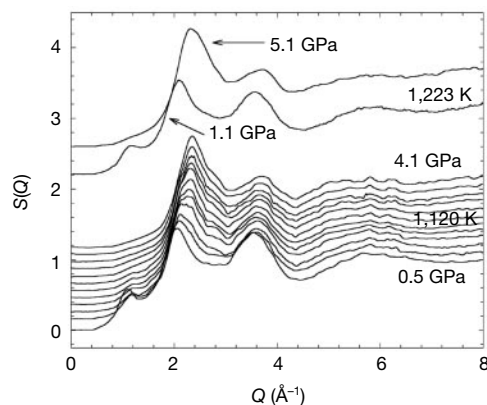


Figure 2 *S*(*Q*) data for liquid GeSe₂. Data are shown for the near-isothermal experiments at 1,121 K from 0.5 to 4.1 GPa. The first sharp diffraction peak (FSDP) is located at $0.9 \text{ \AA}^{-1}$, and shifts to higher *Q* with increasing pressure. The *S*(*Q*) curves were prepared using the Anadol program (see Methods) for extracting normalized intensities by the method of Krogh-Moe and Norman^{29,30}. Analytical searches, through comparison with the ‘large-*Q*’ method of normalization provided the appropriate constants for use in the Fourier transform procedure described by Krogh-Moe and Norman, and implemented by us using the Anadol program.

of the frequency distribution of atoms within neighbouring distances, r , from the centre of an atom). These data, which appear much smoother owing to the rejection of irregularities in the frequency domain, show similar features to the $S(Q)$ curves. The most prominent features are the shift in peak positions to higher values of r , and peak broadening. The first peak is due to the Ge–Se distance, while the second and third peaks correspond to Ge–Ge and Se–Se distances. The first peak shifts from 2.32 to 2.46 Å, while the second peak is less pressure dependent and goes through a maximum near 3 GPa. The average Ge–Se bond distance (about 2.36 Å) in tetragonal GeSe₂, with coordination number 4:2, respectively coordinations of Ge:Se, has been shown to be nearly independent of pressure¹⁶. In comparison, the Ge–Se bond distance is 2.87 Å in ambient-pressure high-temperature GeSe with the rock salt structure (coordination number 6:6). The increasing Ge–Se bond distance shown in Fig. 3 is consistent with an increasing coordination number of Ge and Se. The loss of intermediate-range order shown by the disappearance of the FSDP and the shift in the peaks in $G(r)$ point to a weakening of the covalent character of the Ge–Se bond, and indicate that the liquid structure at high pressure could be modelled by random packing of hard spheres.

The search for polymorphic phase transitions in the liquid or glassy state has been guided by complex thermodynamic behaviour, such as the density maximum in the case of water⁴, and the melting point maximum³ in I, S, Se. The thermodynamic behaviour of supercooled GeSe₂ is particularly interesting, owing to the density minimum discussed above and the melting-point maximum shown in Fig. 1. In the pressure range beyond the melting-point maximum, the liquid structure changes continuously from 2D to 3D. The observation of two different amorphous structures of GeSe₂ (ref. 9), which mimics the 2D and 3D crystalline polymorphs of GeSe₂, suggests that there might be a first-order transition between two GeSe₂ liquids with different density and intermediate-range order²². The transition is most likely to occur in the pressure range near the monoclinic-to-tetragonal phase transition of crystalline GeSe₂, but the critical temperature for the liquid–liquid phase transition may be obscured by the glass transition¹. Future investigations of glassy and supercooled GeSe₂ should elucidate if such a transition exists.

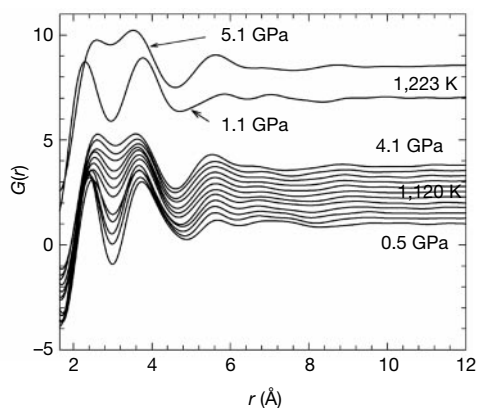


Figure 3 $G(r)$ curves derived for the $S(Q)$ relations shown in Fig. 2. These were obtained by Fourier transform of the $S(Q)$. Care was taken to choose the limits of the Fourier transform so as to ensure that no undue modulations were included in the resulting data due to integration bounds being too high in r , compared with noise levels at high Q . The FSDP was not excluded from the lower bound of the Fourier transform, and no π oscillations are seen to originate from its inclusion. The $G(r)$ curves appear much smoother as irregularities are rejected by the transform in the frequency domain. As pressure is increased, peak broadening and then narrowing is seen. During the transition, at ~ 2.5 GPa, we can see peak widths narrowing again, then increasing again though the effects of different bond compressibilities.

We expect that the present finding—that the structure of liquid GeSe₂ changes significantly with increasing pressure—will stimulate further investigations of the properties of GeSe₂ below the melting line. Both the changes from 2D to 3D character of the liquid below 2.5 GPa, and the loss of the intermediate order above 2.5 GPa, might develop into critical phenomena in the supercooled state, in line with the indications from the glassy state^{9,10}. □

Methods

High-temperature high-pressure *in situ* X-ray diffraction was performed using the Paris-Edinburgh large-volume apparatus installed at the high-pressure beamline (ID30) at the European Synchrotron Radiation Facility. The GeSe₂ sample was prepared as previously described¹⁶. Angle-dispersive diffraction data were collected using a recently developed multi-slit system, which allows sample diffraction patterns to be taken with the minimum of signal from the BN sample capsule, the surrounding furnace and the boron epoxy gasket. Subtraction of the background was made possible by displacing the cell and diffracting from an off-centre position, thus revealing the relative extent of the boron epoxy gasket, furnace and hexagonal BN contribution to the sample scan. The sample-detector distance and spatial distortion were corrected using a Si standard. All 2D patterns were subsequently integrated using Fit2D²³ software. The wavelength was tuned to 0.20215 or 0.26473 Å using an in vacuum Si(111) monochromator. The corrected diffraction patterns were subject to intensity normalization using the Anadol program (version 1; Ph. Faure, CEA-Valduc, 2000) by the atomic scattering factors, and corrections made for incoherent scattering using the coefficients of Pálincas for both Ge and Se (ref. 24). Pressure was determined from scans taken of the hexagonal BN capsule material²⁵, and temperature was determined from furnace power curves that have been calibrated by known fusion curves and an internal cross-calibration method²⁶. The estimated absolute error in the temperature and pressure determination is $\pm 75^\circ\text{C}$ and ± 0.1 GPa. The experiments were conducted by first compressing the sample to the given pressure, followed by increasing the temperature at isobaric conditions. Melting of the sample was clearly seen through the effects of the transition on the furnace power. A diffraction pattern was collected every 100 K below the melting line, and every 25 K near the melting line. The melting temperature at each pressure was determined by the absence of Bragg reflections owing to crystalline GeSe₂ in the recorded patterns. Studies of liquid GeSe₂ were conducted by increasing the pressure to approximately 0.4 GPa followed by heating up to 1,121 K. The temperature was then kept roughly constant while pressurising, based on previous internal calibrations of the furnace behaviour at pressure. Diffraction patterns were collected at pressures between 0.5 and 4.1 GPa.

Received 14 February; accepted 8 October 2001.

- Poole, P., Grande, T., Angell, C. A. & McMillan, P. F. Polymorphism in liquids and glasses. *Science* **275**, 322–323 (1996).
- Katayama, Y. *et al.* First-order liquid-liquid phase transition in phosphorus. *Nature* **403**, 170–173 (2000).
- Brazhkin, V. V., Popova, S. V. & Voloshin, R. N. High-pressure transformations in simple melts. *High Pressure Res.* **15**, 267–305 (1997).
- Poole, P. H., Scortino, F., Essmann, U. & Stanley, H. E. Phase behaviour of metastable water. *Nature* **360**, 324–328 (1992).
- Mishima, O. Liquid-liquid critical point in heavy water. *Phys. Rev. Lett.* **85**, 334–336 (2000).
- Lacks, D. J. First order amorphous-amorphous transformation in silica. *Phys. Rev. Lett.* **84**, 4629–4632 (2000).
- van Thiel, M. & Ree, F. H. High-pressure liquid-liquid phase change in carbon. *Phys. Rev. B* **48**, 3591–3599 (1993).
- Aasland, S. & McMillan, P. F. Density-driven liquid-liquid phase separation in the system $\text{Al}_2\text{O}_3\text{--Y}_2\text{O}_3$. *Nature* **369**, 633–636 (1994).
- Shimada, M. & Dachille, F. Crystallization of amorphous GeSe₂ under pressure. *Inorg. Chem.* **16**, 2094–2097 (1977).
- Prasad, M. V. N., Asokan, S., Parthasarthy, G., Titus, S. S. K. & Gopal, E. S. R. Electrical resistivity studies on Ge–Se glasses at high pressures and low temperatures. *Phys. Chem. Glasses* **34**, 199–202 (1993).
- Grzechnik, A., Grande, T. & Stölen, S. Pressure-induced amorphization of GeSe₂. *J. Solid State Chem.* **141**, 248–254 (1998).
- Ruska, J. & Thurn, H. Change of short-range order with temperature and composition in liquid Ge₂Se₃, as shown by density measurements. *J. Non-Cryst. Solids* **22**, 277–290 (1976).
- Petri, I., Salmon, P. S. & Fischer, H. Defects in a disordered world: The structure of glassy GeSe₂. *Phys. Rev. Lett.* **84**, 2413–2416 (2000).
- Dittmar, V. G. & Schäfer, H. Die Kristallstruktur von Germaniumdiselenid. *Acta Crystallogr. B* **32**, 2726–2728 (1976).
- Grande, T. *et al.* Structural properties of GeSe₂ at high pressures. *J. Solid State Chem.* **145**, 167–173 (1999).
- Grzechnik, A., Stölen, S., Bakken, E., Grande, T. & Mezouar, M. Structural transformations in three-dimensional crystalline GeSe₂ at high pressures and temperatures. *J. Solid State Chem.* **150**, 121–127 (2000).
- Petri, I., Salmon, P. S. & Howells, W. S. Change in topology of the glass forming liquid GeSe₂ with increasing temperature. *J. Phys. Condens. Matter* **11**, 10219–10227 (1999).
- Glazov, V. M., Pavlova, L. M. & Gaev, D. S. Construction of p–T–X diagrams of A^{IV}–Se (A^{IV} = Ge, Sn, Pb) systems based on liquid curvature data at the melting point of congruently melting compounds. *Inorg. Mater.* **20**, 1268–1274 (1984).
- O'Hare, P. A. G. & Zywocinski, A. Thermodynamics of (germanium + selenium): A review and critical assessment. *J. Chem. Thermodyn.* **28**, 459–480 (1996).

20. Massobrio, C., van Roon, F. H. M., Pasquarello, A. & De Leeuw, S. W. Breakdown of intermediate-range order in liquid GeSe₂ at high temperatures. *J. Phys. Condens. Matter* **12**, L697–L704 (2000).
21. Massobrio, C. & Pasquarello, A. Origin of the first sharp diffraction peak in the structure factor of disordered network-forming systems: Layers or voids? *J. Chem. Phys.* **114**, 7976–7979 (2001).
22. Polsky, C. H. et al. Pressure-induced crystallization of vitreous ZnCl₂. *Phys. Rev. B* **61**, 5934–5938 (2000).
23. Hammersley, A. P. et al. Calibration and correction of distortions in two-dimensional detector systems. *Rev. Sci. Instrum.* **66**, 2729–2733 (1995).
24. Pálincas, G. Analytic approximations for the incoherent x-ray intensities of the atoms from Ca to Am. *Acta Crystallogr. A* **29**, 10–12 (1973).
25. Le Godec, Y. et al. in *Science and Technology of High Pressure. Proceedings of AIRAPT-17* 925–928 (eds Manghnani, M. H., Nellis, W. J. & Nicol, M. F.) (Universities Press, Hyderabad, 2000).
26. Crichton, W. A. & Mezouar, M. Non-invasive pressure and temperature estimation in large-volume apparatus by equation-of-state cross-calibration. *High Temp. High Press.* **34**, 235–242 (2002).
27. Stolen, S., Johnsen, H. B., Boe, C. S., Grande, T. & Karlsen, O. B. Stable and metastable phase equilibria in the GeSe₂-Se system. *J. Phase Equil.* **20**, 1–10 (1999).
28. Stolen, S., Grzechnik, A., Grande, T. & Mezouar, M. Anisotropic compressibility and expansivity in layered GeSe₂. *Solid State Commun.* **115**, 249–252 (2000).
29. Krogh-Moe, J. A method for converting experimental x-ray intensities to an absolute scale. *Acta Crystallogr.* **9**, 951–953 (1956).
30. Norman, N. The Fourier transform method for normalizing intensities. *Acta Crystallogr.* **10**, 370–373 (1957).

Acknowledgements

W.A.C. thanks G. Monaco for discussions throughout the project. We thank P.S. Salmon for providing us with neutron-scattering data on liquid GeSe₂ for comparative purposes.

Correspondence and requests for materials should be addressed to W.A.C. (e-mail: crichton@esrf.fr).

Direct splitting of water under visible light irradiation with an oxide semiconductor photocatalyst

Zhigang Zou*, Jinhua Ye†, Kazuhiro Sayama* & Hironori Arakawa*

* Photoreaction Control Research Center (PCRC), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan

† Materials Engineering Laboratory (MEL), National Institute for Materials Science (NIMS), 1-2-1 Sengen, Tsukuba, Ibaraki 305-0047, Japan

The photocatalytic splitting of water into hydrogen and oxygen using solar energy is a potentially clean and renewable source for hydrogen fuel. The first photocatalysts suitable for water splitting¹, or for activating hydrogen production from carbohydrate compounds made by plants from water and carbon dioxide², were developed several decades ago. But these catalysts operate with ultraviolet light, which accounts for only 4% of the incoming solar energy and thus renders the overall process impractical. For this reason, considerable efforts have been invested in developing photocatalysts capable of using the less energetic but more abundant visible light^{3–7}, which accounts for about 43% of the incoming solar energy. However, systems that are sufficiently stable and efficient for practical use have not yet been realized. Here we show that doping of indium-tantalum-oxide with nickel yields a series of photocatalysts, In_{1–x}Ni_xTaO₄ ($x = 0–0.2$), which induces direct splitting of water into stoichiometric amounts of oxygen and hydrogen under visible light irradiation with a quantum yield of about 0.66%. Our findings suggest that the use of solar energy for photocatalytic water splitting might provide a viable source for ‘clean’ hydrogen fuel, once the catalytic efficiency of the semiconductor system has been improved by increasing its surface area and suitable modifications of the surface sites.

The photocatalytic splitting of water using oxide semiconductors is initiated by the direct absorption of a photon, which creates separated electrons and holes in the energy band gap of the material³. These charges can move to the surface of the semiconductor particle and react with water, provided the potential difference of this reaction exceeds 1.23 eV, dictated by the standard potential E^0 of water being –1.23 (standard hydrogen electrode, SHE, pH = 0).

The oxide semiconductor system we used for this reaction is InTaO₄, which crystallizes in the monoclinic wolframite-type structure with space group *P2₁/a*. The structure (Fig. 1a and b) contains TaO₆ and InO₆ octahedra in a unit cell. The InO₆ octahedra share edges to form zigzag chains along the [100] direction, with individual [InO₆]_∞ octahedra chains linked by TaO₆ octahedra into a three-dimensional network. The volumes of the InO₆ and TaO₆ octahedra in InTaO₄ are 13.601 and 10.648 Å³, respectively⁸. Because the ionic radius of Ni²⁺ (0.78 Å) is much smaller than that of In³⁺ (0.92 Å), substituting In³⁺ by Ni²⁺ should reduce the volume of the InO₆ octahedra, and hence the cell volume in InTaO₄. This should in turn affect the direct metal–metal bonding in the crystal, with the In–In distance shortened, resulting in profound alterations of the electronic properties of the compounds^{8,9}.

Single phases of In_{1–x}Ni_xTaO₄ were synthesized by a solid-state reaction at 1,100 °C, using pre-dried In₂O₃, Ta₂O₅ and NiO (99.99% purity) as starting materials. To increase the photocatalytic activity of the materials^{10,11}, we used impregnation with aqueous Ni(NO₃)₂ or RuCl₃ solution to load the oxide semiconductor surface with 1.0 wt% partly oxidized nickel or RuO₂; these loaded materials act as electron traps and hydrogen evolution sites. The Ni-loaded photocatalysts were calcined at 350 °C for 1 h in air and reduced in H₂ atmosphere (200 torr) at 500 °C for 2 h, then treated in O₂ atmosphere (100 torr) at 200 °C for 1 h. The reduction–oxidation treatment¹¹ produced a double-layered structure of metallic Ni and NiO (denoted NiO_y) on the surface of the photocatalyst; this double-layered structure suppresses the backward reaction of water splitting, which is activated by metallic nickel surfaces. The Ru-loaded photocatalysts were calcined at 500 °C for 2 h in air.

The water-splitting experiments were carried out with 0.5 g powdered photocatalyst suspended in 250 ml of pure water in a pyrex glass cell. A 300-W Xe arc lamp was focused through a shutter window and a 420 nm long pass filter was placed on the surface of the cell. The gases evolved were determined by the thermal conductivity detector (TCD) gas chromatograph, which was connected to the glass-made gas circulating line attached to the pyrex glass cell.

The results of the photocatalytic reaction and photophysical parameters are listed in Table 1. The non-doped catalyst, NiO_y/InTaO₄, is active, but the activity was significantly enhanced by Ni doping of InTaO₄. As a typical example, Fig. 2 shows the evolution of H₂ and O₂ from pure water containing suspensions of NiO_y/In_{0.9}Ni_{0.1}TaO₄ and RuO₂/In_{0.9}Ni_{0.1}TaO₄ under visible light irradiation ($\lambda > 420$ nm). The rates of H₂ and O₂ evolution were about 16.6 and 8.3 μmol h^{–1}, respectively, and the quantum yield at 402 nm was estimated to be 0.66% by using an interference filter ($\lambda = 402$ nm; half-width, 15.3 nm). For RuO₂/In_{0.9}Ni_{0.1}TaO₄, the rates of H₂ and O₂ evolution were about 8.7 and 4.3 μmol h^{–1}, respectively. The gas formation rate of the NiO_y loaded sample is about twice as large as that of the RuO₂-loaded sample. The gas evolution stopped when the light was turned off, showing that the reaction is induced by the absorption of visible light and not by tribological processes, such as the so-called mechano-catalysis.

After evacuating the reaction system and re-running the experiment, almost identical gas production rates were achieved in the second as well as third runs. More than 7,200 μmol gases evolved (H₂, about 4,800 μmol; and O₂, about 2,400 μmol) during the course of a 400-h experiment. The catalyst samples remained unchanged during the course of reaction, suggesting that the

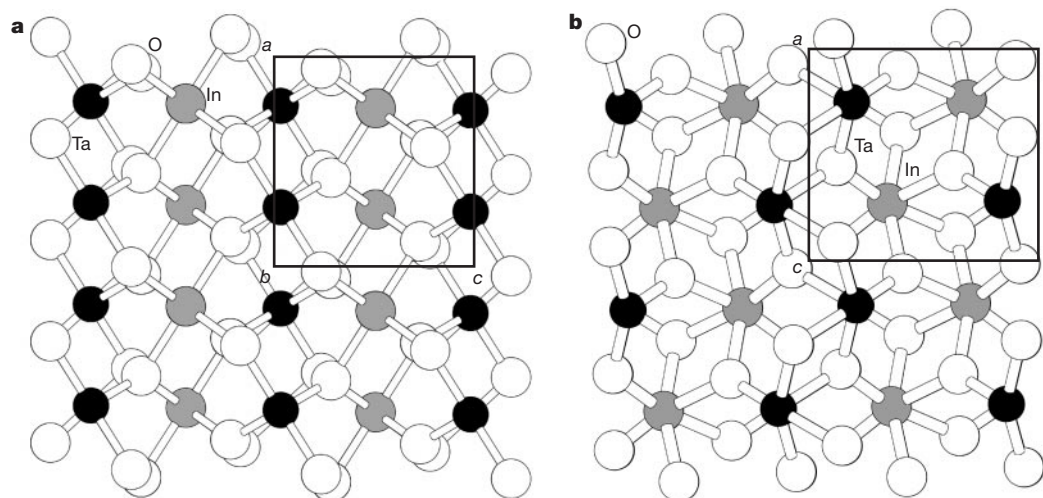


Figure 1 Schematic illustration of the InTaO_4 structure. Shown is the InTaO_4 structure with monoclinic wolframite-type, with the lattice parameters $a = 5.1552(1)$, $b = 5.7751(1)$, $c = 4.8264(1)$ (Å) and $\beta = 91.373(1)^\circ$ obtained from full-profile structure refinement

of XRD data using the Rietveld program REITAN¹². **a**, The view along the b -axis; **b**, the view along the c axis.

photocatalytic activity is stable under visible light irradiation. The turnover number—the ratio of total amount of gas evolved to catalyst (1,412 μmol in our system)—exceeded 5 after 400 h reaction time. The turnover number in terms of reacted electrons relative to the amount of Ni loaded on the surface of the sample and Ni doped in InTaO_4 reached 39 at 400 h reaction time, indicating that the reaction occurs catalytically.

Wavelength dependence of the photocatalytic gas evolution on the photocatalyst was investigated using different cut-off filters. As a comparison, the Pt/TiO_2 photocatalyst (P-25) was measured using the same method. Although photocatalytic activity was observed when using $\text{In}_{1-x}\text{Ni}_x\text{TaO}_4$ and a cut-off filter of $\lambda > 500$ nm, it is much lower than that observed under $\lambda > 420$ nm light irradiation. The rates of H_2 and O_2 evolution were 1.91 and 0.86 $\mu\text{mol h}^{-1}$ on

$\text{In}_{0.9}\text{Ni}_{0.1}\text{TaO}_4$, respectively, under $\lambda > 500$ nm light irradiation. The activity disappeared when the wavelength of light irradiation was larger than 550 nm. The results are in good agreement with the observation of diffuse reflectance spectra (see Fig. 3). By contrast, no photocatalytic activity was obtained on Pt/TiO_2 under visible light irradiation ($\lambda > 420$ nm).

X-ray diffraction analysis showed that there is no observable structural difference between the samples before and after reaction. Full-profile structure refinement of XRD data was performed using the Rietveld program REITAN¹², showing that all samples crystallize in the same wolframite structure, monoclinic with space group $P2_1/a$, and that the lattice parameters decrease along all three axes as the Ni content x is increased, as long as $x < 0.15$. Figure 4 shows the change of V/Z with doping content, where V and Z are cell volume and the number of formulas per cell, respectively. The inset shows the c -axis parameter as a function of Ni content, to illustrate the lattice parameter changes induced by doping. There is a linear decrease of V/Z with increasing Ni content as long as $x < 0.15$, while V/Z has a small expansion when the Ni content $x > 0.15$. In compounds with a Ni-doping content greater than 0.2, an impurity

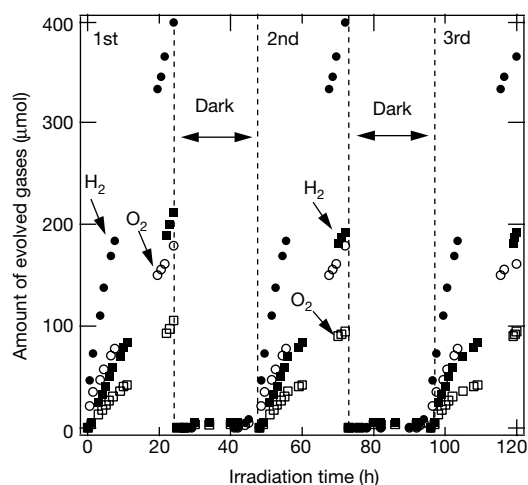


Figure 2 Photocatalytic H_2 and O_2 generation. Shown are the evolution of H_2 and O_2 from pure water using as catalyst a suspension of $\text{NiO}_y/\text{In}_{0.9}\text{Ni}_{0.1}\text{TaO}_4$ (solid circles, H_2 ; open circles, O_2) and $\text{RuO}_2/\text{In}_{0.9}\text{Ni}_{0.1}\text{TaO}_4$ (solid squares, H_2 ; open squares, O_2). Experiments were done using 0.5 g catalyst powder suspended in 250 ml pure water in a pyrex glass cell under visible light irradiation ($\lambda > 420$ nm). Light source: 300-W Xe lamp. The gases evolved were determined by TCD gas chromatograph. The measurement uncertainties were about 0.05%.

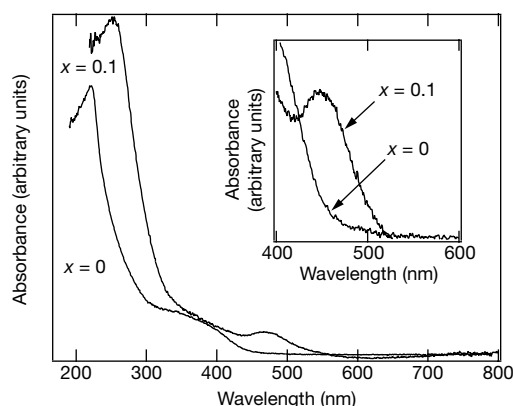


Figure 3 Optical properties of the photocatalyst. The main panel shows the ultraviolet-visible diffuse reflectance spectra of $\text{In}_{1-x}\text{Ni}_x\text{TaO}_4$ ($x = 0$ and 0.1) at room temperature, with the inset providing an expanded view of the spectra in the wavelength region from 400 to 600 nm.

Table 1 Rates of gas evolution and physical properties of the photocatalysts

Photocatalyst	BET† (m ² g ⁻¹)	$t_{\pm}$ ($r_A + r_0$)/ $\sqrt{2(r_B + r_0)}$	Rate of evolution (μmol h ⁻¹)§			
			NiO _x /		RuO ₂ /	
			H ₂	O ₂	H ₂	O ₂
SiO ₂ *			0.0	0.0	0.0	0.0
InTaO ₄	1.35	0.793	3.2	1.1	0.75	0.35
In _{0.95} Ni _{0.05} TaO ₄	0.87	0.790	4.2	2.1	2.0	1.0
In _{0.90} Ni _{0.10} TaO ₄	0.85	0.787	16.6	8.3	8.7	4.3
In _{0.85} Ni _{0.15} TaO ₄	0.82	0.784	8.3	4.1	4.8	2.3
In _{0.80} Ni _{0.20} TaO ₄	0.81	0.780	4.3	0.9	0.8	0.4

* In order to investigate the effect of NiO_x on photocatalytic activity onto the surface of photocatalysts, the 1.0 wt% NiO_x/SiO₂ sample was prepared because SiO₂ does not have photocatalytic activity under visible light irradiation.

† Their BET surface areas were calculated from N₂ isotherms at -196 °C.

‡ t is the tolerance factor for structural stability.

§ NiO_x/ is 1.0 wt% NiO_x loading on the surface of photocatalyst; RuO₂/ is 1.0 wt% RuO₂ loading on the surface of photocatalyst. The rate of gas evolution was calculated for 24 h using 0.5 g catalyst under visible light irradiation $\lambda > 420$ nm.

phase, NiTa₂O₆, appears and increases its volume fraction significantly with increasing Ni content. It is known¹³ that the structural stability of oxides consisting of octahedra such as ABO₃, can be estimated by calculating the tolerance factor defined as $t = (r_A + r_0)/\sqrt{2(r_B + r_0)}$, where r_A , r_B and r_0 are the radii of the respective ions. $0.79 < t < 1.1$ would be the ideal cubic structure. In this case, r_A is a maximum for the non-doped InTaO₄ compound. As shown in Table 1, the t value decreases with increasing Ni content x , the t value becoming smaller than 0.78 when $x > 0.20$, suggesting that the geometrical arrangement in the oxide governs the structural stability.

Figure 3 illustrates the light absorption properties of In_{1-x}Ni_xTaO₄, showing that the visible absorption spectra of these compounds are characteristic of photocatalysts able to respond to visible light. The band gap of these compounds can be estimated from plots of the square root of Kubelka–Munk functions $F(R)$ versus photon energy¹⁴. One of the most characteristic features is that the E_g value (E_g is bandgap energy) is narrowed with Ni doping. The bandgap is changed from 2.6 (non-doped) to 2.3 eV (0.1 Ni-doped). This is considered largely to be a consequence of the Ni 3d level¹⁵. The band structure of NiO is assigned to Ni 3d⁸ and Ni 3d⁹ (ref. 15).

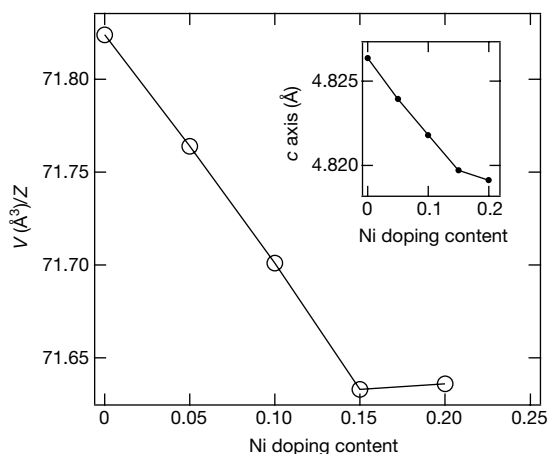


Figure 4 Changes in crystal structure with doping. Shown is the change of V/Z with Ni doping content x , where V and Z are the unit cell volume and the number of formulas per cell, respectively. The inset shows Ni dependence of the c -axis parameter, as an example of the lattice parameter change. All the parameters were obtained from full-profile structure refinements of XRD data using the Rietveld program REITAN¹². The measurement uncertainties were about 0.002 (Å³), for V/Z and 0.0001 (Å) for the c -axis parameter.

The bandgap change in Ni-doped compounds can be attributed to internal transitions in a partly filled Ni d shell. This is supported by the appearance in the Ni-doped compounds of an ultraviolet–visible absorption band at 420–520 nm (see Fig. 3 inset), corresponding to an energy range of about 2.9 to 2.3 eV: we note that the correlation splitting of Ni 3d⁸ and 3d⁹ is 2.4 eV. It is still unknown how a Ni d – d transition could induce electron–hole separation and the generation of hydrogen and oxygen, but it seems clear that the d orbitals play an important role in the photoexcitation and photocatalytic activity observed. In any case, the narrower bandgap will facilitate excitation of an electron from the valence band to the conduction band in the doped oxide semiconductor, thus increasing the photocatalytic activity of the material. □

Received 5 July; accepted 1 October 2001.

- Honda, K. & Fujishima, A. Electrochemical photolysis of water at a semiconductor electrode. *Nature* **238**, 37–38 (1972).
- Kawai, T. & Sakata, T. Conversion of carbohydrate into hydrogen fuel by a photocatalytic process. *Nature* **286**, 474–476 (1980).
- Linsebigler, A. L., Lu, G. & Yates, J. T. Jr Photocatalysis on TiO₂ surfaces: principles, mechanisms, and selected results. *Chem. Rev.* **95**, 735–758 (1995).
- Geoffrey, B. S. & Thomas, E. M. Visible light photolysis hydrogen using sensitized layered metal oxide semiconductors: the role of surface chemical modification in controlling back electron transfer reactions. *J. Phys. Chem. B* **101**, 2508–2513 (1997).
- Kim, Y. II., Salim, S., Huq, M. J. & Mallouk, T. E. Visible light photolysis of hydrogen iodide using sensitized layered semiconductor particles. *J. Am. Chem. Soc.* **113**, 9561–9563 (1991).
- Yoshimura, J., Ebina, Y., Kondo, J. & Domen, K. Visible light induced photocatalytic behavior of a layered perovskite type niobate, RbPb₂Nb₃O₁₀. *J. Phys. Chem.* **97**, 1970–1973 (1993).
- Kudo, A. & Mikami, I. New In₂O₃(ZnO)_m photocatalysts with lamellar structure for visible light induced H₂ or O₂ evolution from aqueous solutions containing sacrificial reagents. *Chem. Lett.* 1027–1028 (1998).
- Zou, Z., Ye, J. & Arakawa, H. Structural properties of InNbO₄ and InTaO₄: correlation with photocatalytic and photophysical properties. *Chem. Phys. Lett.* **332**, 271–277 (2000).
- Zou, Z., Ye, J. & Arakawa, H. Substitution effects of In³⁺ by Al³⁺ and Ga³⁺ on the photocatalytic and structural properties of the Bi₂InNbO₇ photocatalyst. *Chem. Mater.* **13**, 1765–1769 (2001).
- Kim, H. G., Hwang, D. W., Kim, J., Kim, Y. G. & Lee, J. Highly donor-doped (110) layered perovskite materials as novel photocatalysts for overall water splitting. *Chem. Commun.* 1077–1078 (1999).
- Kudo, K. *et al.* Nickel-loaded K₄Nb₆O₁₇ photocatalyst in the decomposition of H₂O into H₂ and O₂: Structure and reaction mechanism. *J. Catal.* **120**, 337–352 (1989).
- Izumi, F. J. A software package for the Rietveld analysis of X-ray and neutron diffraction patterns. *Crystallogr. Ass. Jpn* **27**, 23–31 (1985).
- Machida, M., Yabunaka, J.-i. & Kijima, T. Synthesis and photocatalytic property of layered perovskite tantalates, RbLnTa₂O₇ (Ln = La, Pr, Nd, and Sm). *Chem. Mater.* **12**, 812–817 (2000).
- Kim, Y. II., Atherton, S., Brigham, E. S. & Mallouk, T. E. Sensitized layered metal oxide semiconductor particles for photochemical hydrogen evolution from nonsacrificial electron donors. *J. Phys. Chem.* **97**, 11802–11810 (1993).
- Dare-Edwards, M. P., Goodenough, J. B., Hammett, A. & Nicholson, N. D. Photoelectrochemistry of nickel (II) oxide. *J. Chem. Soc. Faraday Trans. 77*, 643–661 (1981).

Acknowledgements

This work was supported in part by the COE project, Japan Ministry of Education and Science.

Correspondence and requests for materials should be addressed to Z.Z. (e-mail: z.zou@aist.go.jp) or H.A. (e-mail: h.arakawa@aist.go.jp).

Growth processes in teeth distinguish modern humans from *Homo erectus* and earlier hominins

Christopher Dean*, Meave G. Leakey†, Donald Reid‡, Friedemann Schrenk§, Gary T. Schwartz||, Christopher Stringer¶ & Alan Walker#

* Evolutionary Anatomy Unit, Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK

† Department of Palaeontology, National Museums of Kenya, PO Box 40658, Nairobi, Kenya

‡ Oral Biology, Dental School, Framlington Place, Newcastle upon Tyne NE2 4BW, UK

§ Forschungsinstitut Senckenberg, Palaeoanthropologie, Senckenberganlage 25, D-60325 Frankfurt am Main, Germany

|| Department of Anthropology, The George Washington University, 2110 G Street, NW, Washington DC 20052, USA

¶ Human Origins Group, Department of Palaeontology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK

Anthropology Department, 409 Carpenter Building, Pennsylvania State University, University Park, Pennsylvania 16802, USA

A modern human-like sequence of dental development, as a proxy for the pace of life history, is regarded as one of the diagnostic hallmarks of our own genus *Homo*^{1–3}. Brain size, age at first reproduction, lifespan and other life-history traits correlate tightly with dental development^{4–6}. Here we report differences in enamel growth that show the earliest fossils attributed to *Homo* do not resemble modern humans in their development. We used daily incremental markings in enamel to calculate rates of enamel formation in 13 fossil hominins and identified differences in this

key determinant of tooth formation time. Neither australopiths nor fossils currently attributed to early *Homo* shared the slow trajectory of enamel growth typical of modern humans; rather, both resembled modern and fossil African apes. We then reconstructed tooth formation times in australopiths, in the ~1.5-Myr-old *Homo erectus* skeleton from Nariokotome, Kenya⁷, and in another *Homo erectus* specimen, Sangiran S7-37 from Java⁸. These times were shorter than those in modern humans. It therefore seems likely that truly modern dental development emerged relatively late in human evolution.

Ameloblasts secrete enamel matrix in a circadian manner^{9–11} and the resulting daily enamel increments can be used as a chronometer of tooth growth and dental development^{11–16}. Occasionally, these increments can be imaged in fossil hominins on naturally fractured tooth surfaces with scanning electron microscopy, with confocal microscopy of the subsurface enamel, or more predictably with polarizing light microscopy of ground sections. We identified regions in enamel that showed a well preserved record of daily enamel cross-striations in 13 teeth or tooth fragments of specimens firmly attributed to three species of early *Homo*, to four species of australopith and one Neanderthal. Cumulative counts of daily increments were recorded at 100-µm intervals along prisms running through occlusal enamel, providing a continuous record of enamel formation rates.

Prisms run in a sinuous manner through enamel, and this influences cumulative enamel formation rate. Some of the observed variation in rate within each taxon results from differing degrees of enamel decussation (how much prisms deviate from a straight path) between individuals. The samples of extant teeth included canines, premolars and molars, so the trajectories are apparently independent of external tooth morphology, tooth size and enamel thickness. We compared enamel growth rates in fossil hominins with those in modern humans, modern African great apes and with two teeth attributed to the African Miocene stem hominoid, *Proconsul nyanzae* (Fig. 1). Modern human enamel develops along a slower trajectory

Table 1 Anterior crown formation times

Taxon	Tooth type (n)	Occlusal enamel formation times in days (95% CL)	Mean perikymata count (s.d.)	Estimated mean crown formation times (days)			Modern human crown formation time (s.d.) in days
				Periodicity 8 d	Periodicity 9 d	Periodicity 10 d	
<i>Homo sapiens</i>	UI1 (19)	289 (282–296)	165 (21)				1,708 (50)
	UI2 (16)	274 (266–282)	134 (16)				1,478 (49)
	UC (39)	355 (347–363)	148 (19)				1,672 (55)
	LI1 (15)	256 (245–267)	133 (11)				1,309 (50)
	LI2 (13)	212 (200–224)	130 (19)				1,376 (46)
	LC (13)	348 (332–364)	199 (22)				2,066 (73)
<i>Australopithecus</i>	UI1 (5)	173 (134–205)	123 (12)	1,157	1,280	1,403	
	UI2 (6)	163 (126–194)	109 (16)	1,035	1,144	1,253	
	UI1 (4)	220 (170–258)	122 (24)	1,196	1,319	1,440	
	LI1 (4)	136 (104–164)	116 (11)	1,064	1,180	1,296	
	LI2 (4)	127 (96–153)	122 (12)	1,103	1,225	1,347	
	LC (5)	216 (167–253)	143 (8)	1,360	1,503	1,646	
<i>Paranthropus</i>	UI1 (7)	173 (134–205)	83 (12)	837	920	1,003	
	UI2 (7)	163 (126–194)	85 (6)	843	928	1,013	
	UC (2)	220 (170–258)	112 (11)	1,116	1,228	1,340	
	LI1 (9)	136 (104–164)	78 (13)	760	838	916	
	LI2 (3)	127 (96–153)	90 (10)	847	937	1,027	
	LC (6)	216 (167–253)	103 (7)	1,040	1,143	1,246	
KNM-WT 15000	UI1 (2)	212 (169–256)	94	964	1,058	1,152	
	UI2 (2)	201 (160–241)	96	969	1,065	1,161	
	UC (1)	266 (209–323)	100	1,066	1,166	1,266	
	LI1 (1)	169 (135–203)	96	937	1,033	1,129	
	LI2 (2)	157 (126–189)	92	893	985	1,077	
	LC (1)	262 (206–317)	110	1,142	1,252	1,362	
KNM-ER 820	LI2 (1)	162 (133–191)	113	1,066	1,179	1,292	
KNM-ER 1590	UI1 (1)	219 (179–258)	114	1,131	1,245	1,359	
	UC (1)	273 (220–324)	127	1,289	1,416	1,543	
KNM-ER 808	UI2 (1)	207 (169–244)	97	983	1,080	1,177	
SK 27	UC (1)	273 (220–324)	153	1,497	1,650	1,803	
Sangiran 4	UC (1)	273 (220–324)	138	1,377	1,515	1,653	

Crown formation time equals the sum of occlusal and lateral enamel formation time. Occlusal enamel formation times for modern humans were calculated directly²⁰; those for fossils were estimated on the basis of average modern human enamel thickness data²⁰ and the regression equations in Fig. 1. Lateral enamel formation times in the human sample were calculated from long-period stria counts of known periodicity²⁰. Lateral enamel formation times in the fossil samples were calculated from perikymata counts made in reflected light with a stereo binocular microscope on high-resolution replicas of anterior teeth attributed to *Australopithecus* (n = 28) and *Paranthropus* (n = 34). Replicas were sputter coated with 20 nm of gold to facilitate this. Repeated inter- and intra-observer counts were made with less than 5% error. Estimates of crown formation time are given for perikymata counts in each tooth type at three presumed modal values for periodicity (8, 9 and 10 days). Estimates for teeth from six individuals attributed to early *Homo* from sites in East and southern Africa and Sangiran, Java, are also given.

because the earliest-formed enamel, closest to the enamel dentine junction, is secreted in smaller increments for a longer period. None of the trajectories of enamel growth in apes, australopiths or fossils attributed to *Homo habilis*, *Homo rudolfensis* or *H. erectus* falls within that of the sample from modern humans.

Enamel is thin in modern African apes¹⁷ but thick in hominins^{18,19}. Occlusal enamel thickness is a simple function of the rate of secretion and the time that secretory ameloblasts continue to form enamel matrix. These trajectories reveal a common developmental basis for the way in which African apes grow thin enamel and early hominins grew thick enamel. Humans form thick enamel along a different developmental trajectory, so thick enamel in modern humans and in early fossil hominins are not homologous.

Radiographs, as well as direct observations of developing teeth, show that the sequence of key events during tooth growth in *H. erectus* was identical to that of modern humans^{3,20–22}. It has been assumed that the timescale of dental development was also identical. However, because the underlying growth processes in enamel differ, this now seems unlikely. An identical sequence of tooth development in modern humans and *H. erectus* nevertheless demonstrates that any shift in timing affected all teeth in the same way and to the same degree. To further explore the timing of crown and root formation we used perikymata on the well preserved anterior dentition of the Nariokotome specimen, KNM-WT 15000, and incremental markings in enamel and dentine in the posterior dentition of Sangiran S7-37.

Perikymata are the surface manifestation of long-period striae of Retzius within enamel and have a modal periodicity of 9 days (range 6–11 days) in great apes and humans^{10,23,24}. They provide a record of how lateral tooth enamel grows in height after the completion of

occlusal enamel. The sum of occlusal and lateral enamel formation times equals the total crown formation time. We calculated crown formation times in well preserved fossil anterior teeth of hominins from sites in East and southern Africa and compared them with those of KNM-WT 15000 and with modern human teeth (Table 1). We then made histological sections through the upper first permanent molar and second premolar of Sangiran S7-37 and used counts of incremental markings in enamel and dentine to reconstruct a timescale for crown and root formation. Estimates of crown formation times for all anterior tooth types in KNM-WT 15000 fall consistently among those for australopiths and are shorter than those in our modern human sample (Fig. 2a). Data for the Sangiran specimen (Fig. 2b) provide further evidence for faster rates of dental development and earlier estimates for tooth emergence times in the posterior dentition.

An understanding of developmental processes can provide powerful insights into the evolutionary history of adult morphologies^{25,26}. Here we show that an apparently similar adult character, enamel thickness, can arise through subtly different generative developmental processes. Despite considerable variation in external tooth morphology, early fossil hominins share a common and fundamental enamel growth trajectory with the African ape clade that is derived in modern humans.

It is now generally held that a prolonged life-history schedule reflects a reduction in the mortality rate of adults, triggered perhaps by behavioural, dietary or other changes⁶. An increase in brain size (and cognitive ability) is associated with this reduction, but does not necessarily drive it, even in the human lineage⁶. The size of key brain components associated with learning and cognition correlates with the timing of dental development in primates^{4,5} as the cost in time needed to grow and learn to use a larger brain increases. In this

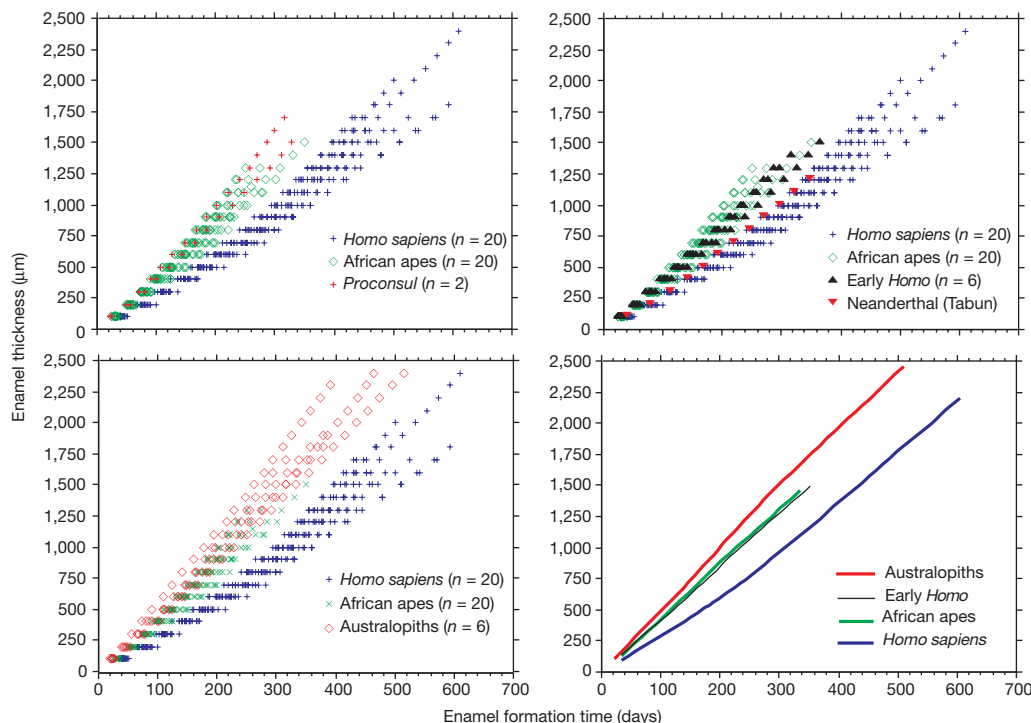


Figure 1 Scatterplots with Lowess regressions of enamel formation rates for samples of hominins. The trajectory for modern human permanent teeth is distinct from all ape and fossil samples. Only the Neanderthal (Tabun specimen C1) falls within the human sample. Plots for australopiths overlap extensively with African apes and early *Homo* but have a slightly faster average rate of enamel growth. This may be an artefact of the way fossil groups are defined here or a result of prisms with less decussation in some australopiths. In all cases the coefficient of determination R^2 was greater for second-order polynomials than for linear least squares regressions. Where y is time of

enamel formation (days) and x is enamel thickness (μm), for 20 modern humans, $y = 8.70 + 0.37x - 0.00005x^2$ ($R^2 = 0.97$, standard error, s.e. = 0.1, $P < 0.0001$). For 20 African apes, $y = 8.68 + 0.25x - 0.00003x^2$ ($R^2 = 0.97$, s.e. = 0.01, $P < 0.0001$). For six australopiths, $y = 6.64 + 0.21x - 0.00001x^2$, ($R^2 = 0.97$, s.e. = 0.01, $P < 0.0001$). For six early *Homo* specimens, $y = 3.76 + 0.26x - 0.00002x^2$, ($R^2 = 0.99$, s.e. = 0.01, $P < 0.0001$). For six plots derived from three teeth attributed to *H. ergaster/erectus*, $y = 3.70 + 0.27x - 0.00003x^2$ ($R^2 = 0.99$, s.e. = 0.01, $P < 0.0001$).

context a slower trajectory of enamel growth in permanent teeth, one of the basic determinants of tooth formation time, can be regarded as a life-history attribute associated with the extended, or prolonged, growth period of modern humans. The first evidence for a shift in enamel growth rates in the hominin fossil record seems to be with the origin of larger-brained Neanderthals (at least by 100 kyr ago; see Tabun specimen C1 in Fig. 1) and modern humans.

Enamel microanatomy has previously been important in attributing enigmatic early hominin specimens to *Australopithecus* or *Homo*^{18,27,28}. In a recent review of the key attributes that might be used to define the genus *Homo*², it has been recommended that fossils currently attributed to *H. habilis* and *H. rudolfensis* be transferred (or returned) to *Australopithecus*. Although we present no data for *H. habilis* from Tanzania or South Africa (only for *H. habilis*, KNM-ER 1805, and *H. rudolfensis*, KNM-ER 1482 and KNM-ER 1802, from Kenya (all ref. 27)), the data presented here support this. Our results do not support the notion that the sequence of tooth development in *H. erectus* indicates that the timing of tooth development events was like that in modern humans, even though other sound nondental criteria suggest that the assignation to *Homo* is correct^{2,7}. Indeed, we are cautious about assuming that an apparently modern-human-like sequence of tooth development in any hominin fossil necessarily implies an extended period of growth¹⁻³. Although it seems clearer that the hallmark of modern human dental development is a slow trajectory of enamel growth combined with an initial slow rate of tooth root extension²², it may still be premature to incorporate details of dental development into definitions of the genus *Homo*².

Permanent molar emergence times are especially significant in

primates because they correlate with many life-history traits⁴⁻⁶. Our data do not allow us to reconstruct a direct age for the emergence of molars M1 or M2, or an age at death for the Nariokotome or Sangiran specimens. Nonetheless, we speculate on the basis of the data presented in Fig. 2 that M1 emergence occurred around 4 years of age in KNM-WT 15000, close to the time of enamel completion in the lower canines, as it does in modern humans (and *Paranthropus*¹⁴). This age is in broad agreement with another estimate (4.5 years) for average M1 emergence in African *Homo erectus*^{7,29} on the basis of mean endocranial capacity (826 ml) and implies an age at death for KNM-WT 15000 closer to 8 than 12 years of age^{20,29}. Histological reconstruction of dental development in Sangiran S7-37 (Fig. 2b) suggests that gingival emergence of M1 was around 4.4 years and around 7.6 years for premolar P4 and M2 in this individual. If correct, these estimates of molar emergence times have shifted a little, in step with brain size, from those known for African great apes and australopiths. Nevertheless, it now seems increasingly likely that a period of development truly like that of modern humans arose after the appearance of *H. erectus*, when both brain size and body size were well within the ranges known for modern humans. □

Methods

Data collected

The data shown in Fig. 1 were collected by scanning electron microscopy of the naturally fractured surface of KNM-ER 733D (premolar attributed to *Paranthropus boisei*¹²), by confocal microscopy of the subsurface enamel of KNM-ER 1802 (M3 attributed to *H. rudolfensis*²⁷), and by polarizing light microscopy of ground sections of the following samples: SK 63 (lower canine attributed to *Paranthropus robustus*¹⁴); KNM-KP 30748 and KNM-KP 30749 (both molars attributed to *Australopithecus anamensis*); KNM-WT 17000

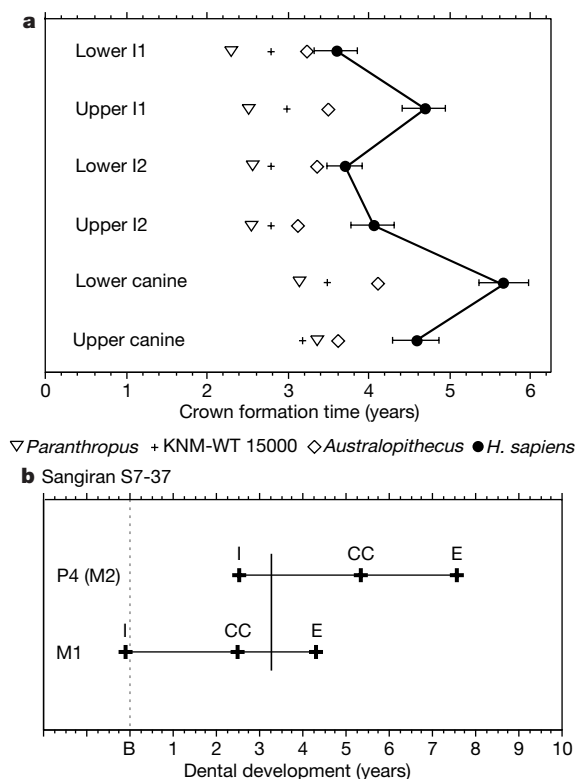


Figure 2 Timing of dental development in hominins. **a**, Crown formation times in hominins. The data are taken from Table 1 and exclude any time between birth and initial mineralization. Those for fossils are based on a perikymata periodicity of 9 days. Estimates of crown formation times for KNM-WT 15000 fall within the australopith range (despite having tall crowns that contrast markedly with smaller *Paranthropus* crowns). The data for modern humans are joined by a line to emphasize the long crown formation times for upper central incisors (UI1) and lower canines. Error bars are two standard deviations.

b, Estimates of the timing of initiation (I) of tooth mineralization in Sangiran S7-37, together with crown completion (CC) and root formed at gingival emergence (E), are shown for the first upper permanent molar (M1) and upper premolar (P4). We assume that M2, which was not preserved, was developmentally nearly identical to P4. Birth (B) is indicated. A strong accentuated line in both M1 and P4 (short vertical line) occurred 3.3 years into postnatal development and allowed the development of these teeth to be cross-matched. See Methods for details of calculation.

(premolar attributed to *Paranthropus aethiopicus*); KNM-ER 801 (molar attributed to *P. boisei*²⁷); KNM-ER 1805E (premolar attributed to *H. habilis*²⁷); KNM-ER 1482B (molar attributed to *H. rudolfensis*²⁷); KNM-ER 809B (molar attributed to *H. ergaster*²⁷); KNM-ER 3733 (lower right P4 attributed to *H. ergaster*²⁷); Sangiran S7-37 (upper right M1 and P4 attributed to *H. erectus*); and Tabun C1 (fragment of lower left first molar metaconid; attributed to Neanderthal).

Dental development in Sangiran S7-37

To estimate the timing of dental development in Sangiran S7-37 (Fig. 2), we counted the number of daily increments in the protocone and paracone of M1, which were equal. However, as the paracone initiates before the protocone, a month or so before birth, we added 30 days of prenatal and 30 days of postnatal enamel formation time, presumed lost through wear and/or plane of section. Total crown formation time in M1 was then 2.5 years to the mesiobuccal cervix. A strong accentuated line in both M1 and P4 (short vertical line in Fig. 2b) occurred 3.3 years into postnatal development and allowed the development of these teeth to be cross-matched. P4 mineralization initiated about 18 days after M1 crown completion. P4 crown formation time took 2.7 years. Root extension rates were calculated using counts and measurements of daily incremental markings in root dentine and averaged $10.7 \mu\text{m}^{-1}$ in M1 and $11 \mu\text{m}^{-1}$ in P4 (7–8 mm of root growth over 3 years in a modern human M1 would extend at $6.4\text{--}7.3 \mu\text{m}^{-1}$). We observed about 8 mm of root formed below the buccal cervix of M1 and about 10 mm in M2 in hominin fossils^{7,14} where these teeth were just in functional occlusion with wear (see also other fossil hominins, for example LH2 from Laitoli, Tanzania and Taung from South Africa). Thus, at gingival emergence we expect there would have been about 1 mm less root formed. On this basis we estimate that gingival emergence for M1 occurred at about 4.4 years of age and for P4 (M2) at about 7.6 years of age in Sangiran S7-37.

Received 13 July; accepted 1 October 2001.

1. Bermudez De Castro, J.-M. *et al.* A modern human pattern of dental development in Lower Pleistocene hominids from Atapuerca-TD6 (Spain). *Proc. Natl Acad. Sci. USA* **96**, 4210–4213 (1999).
2. Wood, B. & Collard, M. The human genus. *Science* **284**, 65–71 (1999).
3. Moggi-Cecchi, J. in *The Origin of Humankind* (eds Aloisi, M., Battaglia, B., Carafoli, E. & Danieli, G. A.) 35–50 (IOS, Amsterdam, 2000).
4. Smith, B. H. Dental development as a measure of life history in primates. *Evolution* **43**, 683–688 (1989).
5. Allman, J. & Hasenstaub, A. Brains, maturation times, and parenting. *Neurobiol. Aging* **20**, 447–454 (1999).
6. Kelley, J. in *Human Evolution through Developmental Change* (eds McNamara, K. J. & Minugh-Purvis, N.). (Johns Hopkins Univ. Press, Baltimore, in the press).
7. Walker, A. & Leakey, R. *The Nariokotome Homo erectus Skeleton* (Harvard Univ. Press, Cambridge, Massachusetts, 1993).
8. Grine, F. E. & Franzen, J. L. Fossil hominid teeth from the Sangiran Dome (Java, Indonesia). *Courier Forschungsinstitut Senckenberg* **171**, 75–103 (1994).
9. Bromage, T. G. Enamel incremental periodicity in the pig-tailed macaque: A polychrome fluorescent labelling study of dental hard tissues. *Am. J. Phys. Anthropol.* **86**, 205–214 (1991).
10. FitzGerald, C. M. Do enamel microstructures have regular time dependency? Conclusions from the literature and a large scale study. *J. Hum. Evol.* **35**, 371–386 (1998).
11. Antoine, D., Dean, C. & Hillson, S. in *Dental Morphology 1998* (eds Mayhall, J. T. & Heikkinen, T.) 48–55 (Oulu Univ. Press, Finland, 1999).
12. Beynon, A. D. & Dean, M. C. Crown formation time of a fossil hominid premolar tooth. *Arch. Oral Biol.* **32**, 773–780 (1987).
13. Beynon, A. D., Dean, M. C., Leakey, M. G., Reid, D. J. & Walker, A. Comparative dental development and microstructure of *Proconsul* teeth from Rusinga Island, Kenya. *J. Hum. Evol.* **35**, 163–209 (1998).
14. Dean, M. C., Beynon, A. D., Thackeray, J. F. & Macho, G. A. Histological reconstruction of dental development and age at death of a juvenile *Paranthropus robustus* specimen, SK 63, from Swartkrans, South Africa. *Am. J. Phys. Anthropol.* **91**, 401–419 (1993).
15. Boyde, A. in *Primate Life History and Evolution* (ed. DeRousseau, J.) 229–267 (Wiley-Liss, New York, 1990).
16. Risnes, S. Growth tracks in enamel. *J. Hum. Evol.* **35**, 331–350 (1998).
17. Schwartz, G. T. Taxonomic and functional aspects of the patterning of enamel thickness distribution in extant large-bodied hominoids. *Am. J. Phys. Anthropol.* **111**, 221–240 (2000).
18. Beynon, A. D. & Wood, B. A. Variations in enamel thickness and structure in East African hominids. *Am. J. Phys. Anthropol.* **70**, 177–193 (1986).
19. Grine, F. E. & Martin, L. B. in *Evolutionary History of the Robust Australopithecines* (ed. Grine, F. E.) 3–42 (Aldine de Gruyter, New York, 1988).
20. Smith, B. H. Patterns of dental development in *Homo*, *Australopithecus*, *Pan* and *Gorilla*. *Am. J. Phys. Anthropol.* **94**, 307–325 (1994).
21. Beynon, A. D. & Dean, M. C. Distinct dental development patterns in early fossil hominids. *Nature* **335**, 509–514 (1988).
22. Dean, M. C. Progress in understanding hominoid dental development. *J. Anat.* **197**, 77–101 (2000).
23. Bromage, T. G. & Dean, M. C. Re-evaluation of the age at death of immature fossil hominids. *Nature* **317**, 525–527 (1985).
24. Schwartz, G. T., Reid, D. J. & Dean, M. C. Developmental aspects of sexual dimorphism in hominoid canines. *Int. J. Primatol.* **22**, 837–860 (2001).
25. Lovejoy, C. O., Cohn, M. J. & White, T. D. Morphological analysis of the mammalian postcranium: A developmental perspective. *Proc. Natl Acad. Sci. USA* **96**, 13247–13252 (1999).
26. Jernvall, J. & Han-Sung, J. Genotype, phenotype and developmental biology of molar tooth characters. *Yearb. Phys. Anthropol.* **43**, 171–190 (2000).
27. Wood, B. A. *Hominid Cranial Remains* Koobi Fora Research Project Vol. 4. (Clarendon, Oxford, 1991).
28. Ramirez Rozzi, F. V. Can enamel microstructure be used to establish the presence of different species of Plio-Pleistocene hominids from Omo, Ethiopia? *J. Hum. Evol.* **35**, 543–576 (1998).
29. Smith, B. H. & Tompkins, R. L. Towards a life history of the Hominidae. *Ann. Rev. Anthropol.* **24**, 257–279 (1995).
30. Reid, D. J. & Dean, M. C. The timing of linear hypoplasias on human anterior teeth. *Am. J. Phys. Anthropol.* **113**, 135–139 (2000).

Acknowledgements

We thank The Government of Kenya; The National Museums of Kenya; Forschungsinstitut Senckenberg, Frankfurt am Main, Germany; the Natural History Museum, London; and F. Thackeray of the Transvaal Museum, South Africa for access to fossil material. We thank D. Antoine, B. Berkovitz, D. Beynon, D. Clements, C. FitzGerald, L. Humphrey, J. Jernvall, J. Kelley, C. Kiarie, R. Krusynski, D. Lieberman, G. Macho, P. O'Higgins, J. Pendjiky, F. Ramirez Rozzi, H. Smith, P. Smith, F. Spoor, P. Walton and B. Wood for their help. This research was enabled by research grants to C.D. from the Royal Society and the Leverhulme Trust.

Correspondence and requests for materials should be addressed to C.D. (e-mail: ucgacd@ucl.ac.uk).

Individual recognition in mice mediated by major urinary proteins

Jane L. Hurst*, Caroline E. Payne*, Charlotte M. Nevison*, Amr D. Marie†, Richard E. Humphries*, Duncan H. L. Robertson†, Andrea Cavaggoni‡ & Robert J. Beynon†

* Department of Veterinary Clinical Science and Animal Husbandry, University of Liverpool, Leahurst, Neston CH64 7TE, UK

† Department of Veterinary Preclinical Sciences, University of Liverpool, Liverpool L69 3BX, UK

‡ Dipartimento di Anatomia e Fisiologia Umana, Università di Padova, 35100 Padova, Italy

The ability to recognize individuals is essential to many aspects of social behaviour, such as the maintenance of stable social groups, parent–offspring or mate recognition, inbreeding avoidance and the modulation of competitive relationships. Odours are a primary mediator of individuality signals among many mammals¹. One source of odour complexity in rodents, and possibly in humans, resides in the highly polymorphic major histocompatibility complex (MHC)². The olfactory acuity of mice³ and rats⁴ allows them to distinguish between the urinary odours of congenic strains differing only in single genes within the MHC, although the chemical mediators or odorants are unknown. However, rodent urine also contains a class of proteins, termed major urinary proteins (MUPs)⁵, that bind and release small volatile pheromones^{6,7}. We have shown that the combinatorial diversity of expression of MUPs among wild mice might be as great as for MHC, and at protein concentrations a million times higher⁸. Here we show in wild house mice (*Mus domesticus*) that urinary MUPs play an important role in the individual recognition mechanism.

The only known function of MUPs is in chemical signalling. MUPs of male mice bind volatile signalling pheromones and release them slowly from urinary scent marks⁹. These volatiles are attractive to male^{10,11} and female¹² mice, stimulate oestrus in prepubertal¹³ and adult^{14,15} females, and stimulate aggression between males¹⁶. In addition, the urinary proteins themselves stimulate increased competitive scent marking¹⁰ and, if derived from a male of an unfamiliar strain, block pregnancy in females¹⁷. MUPs are expressed by both dominant and subordinate male mice¹⁸ and both urine types stimulate increased scent marking by competitive males but not by subordinate males^{18,19}. MUPs are encoded by a multigene family on chromosome 4 (ref. 20), and there are multiple alleles at each locus. The urinary MUPs are readily analysed²¹, and it has become clear that MUPs in the urine of wild house mice exhibit a very high level of polymorphism. Individual mice each express a combination of about 7–12 MUPs and we have found many different MUP patterns, even among mice captured from the same population²². It is difficult to reconcile such molecular diversity with a simple role of

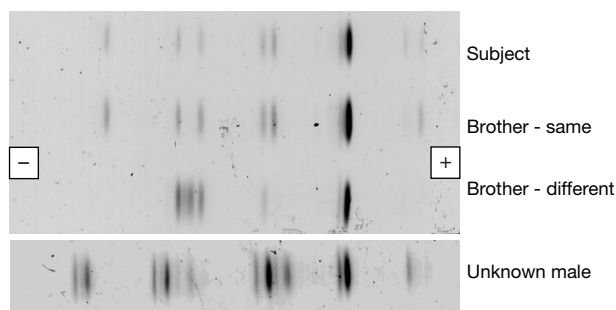


Figure 1 Urinary MUP types. MUPs are readily resolved by isoelectric focusing in narrow range (pH 4.2–4.9) immobilized gradient gels (AP Biotech, UK). Urine (5 μ l of a 1 in 10 dilution) was focused for 15 kV h at 10 °C and stained with Coomassie blue. The overall pattern of bands is the MUP type, and the figure shows representative MUP types of a test subject, of a brother with the same MUP type, of a brother with a different MUP type and the MUP type of an unfamiliar male.

ligand binding and release, and we have previously suggested that the polymorphism in this class of proteins contribute to the individuality signals in urine deposits^{8,10,19}.

We therefore conducted two sets of tests to establish whether differences in MUP profile allow mice to distinguish their own scent from that of other males. The wild house mice were derived from outbred crosses between animals captured from geographically separated populations, to maintain the normal genetically heterogeneous background of wild mice. We also examined natural responses rather than a trained ability to discriminate, making use of the fact that when male mice encounter scent marks in their territory from another male, they spend longer investigating another male's scent than their own and increase their own rates of urine mark deposition to counter-mark the competitor's scent^{10,18,23}. We used matched-pair *t*-tests and Wilcoxon matched-pair exact tests respectively to assess the specific hypothesis of increased investigation and scent marking towards urine stimuli from other males.

In the first set of tests, we compared the responses of the mice to

their own urine, to urine from an unrelated male of different MUP type, and to urine from sibling males of the same or different MUP type as the subject (Fig. 1). In each test, stimulus urine was introduced into a male's home enclosure on one of two squares of absorbent paper for comparison with a control test in which both squares were marked only with water. The second square, marked only with water, also allowed us to examine whether increased scent marking was apparent only in the immediate vicinity of a stimulus or was distributed more widely around the home area. First we confirmed that males responded to the urine marks of an unrelated male but not to their own scent marks (Fig. 2). As expected, males spent more time investigating urine from an unrelated male than water-marked squares in the control test ($t_7 = -2.72$, $P < 0.025$) and deposited more scent marks on the unrelated urine square (Wilcoxon matched-pair exact test, $z = -2.38$, $P < 0.005$). In contrast, a square marked with their own urine stimulated no more investigation ($t_6 = -0.61$, not significant, NS) or scent marks ($z = -0.28$, NS) than water-marked squares in the control test. The scent-marking response induced by unrelated male urine also increased on the water-marked square presented simultaneously (number of scent marks on water-marked tile in unrelated male urine test compared with control test, $z = -2.52$, $P < 0.005$). Thus, scent marking was measured as the total number of marks deposited on both introduced squares in each test for all further analyses. Wilcoxon matched-pair exact tests confirmed that males deposited significantly more total marks in response to unrelated male urine than in either the control ($z = -2.55$, $P < 0.005$) or own urine test ($z = -2.19$, $P < 0.025$) whereas their own urine did not stimulate an increase in total scent marking ($z = 0.65$, NS; Fig. 2c). Investigation was directed towards the urine stimulus only (Fig. 2a), and thus only investigations of the urine-marked squares were compared.

To examine whether MUP type was important in recognizing scent marks as different from own, we compared the responses of the mice to their own urine and to urine from brothers of the same or different MUP type (Fig. 1). Urine from a brother of different MUP type stimulated significantly more investigation ($t_6 = 2.99$, $P < 0.025$) and scent marks ($z = -2.67$, $P < 0.005$) than own urine (Fig. 2), showing clear recognition of the brother's scent

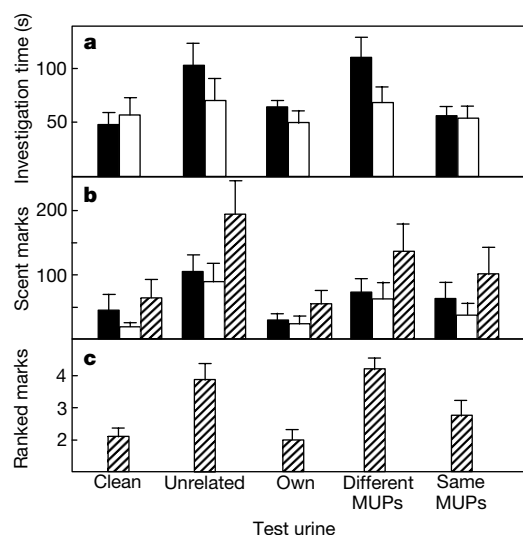


Figure 2 The effect of MUP type on investigation and countermarking of urine marks. **a**, **b**, Investigation time (**a**) and scent marking (**b**) by adult male wild house mice on paired stimulus squares streaked with urine (filled bars) or water (open bars) in five separate tests. Cross-hatched bars show scent marks totalled for each test. **c**, Rank of total scent marks deposited on both stimulus squares across the five tests. Data are means $\pm$ standard error of the mean, s.e.m.

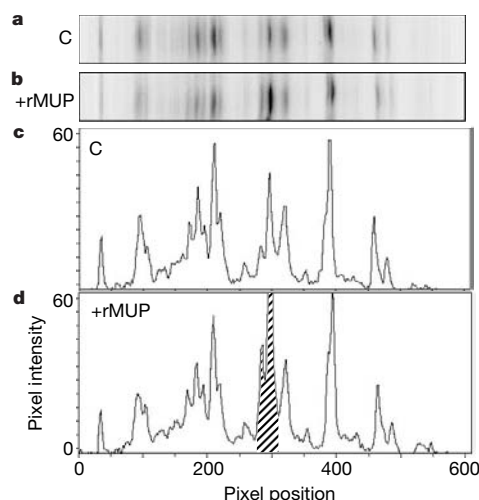


Figure 3 Modification of MUP type by addition of recombinant MUP. A male's own urine sample (C) was modified by addition of recombinant MUP (+rMUP) to a level of 20% of the total protein. Isoelectric focusing (**a**, **b**), followed by densitometry (**c**, **d**) was used to analyse the modification of the urine MUP type by the added protein (a representative example is shown). The cross-hatched area of the +rMUP densitometric trace highlights the change in protein profile elicited by the added rMUP.

marks. Brother's urine of the same MUP type failed to stimulate any more investigation ($t_6 = 0.34$, NS) or scent marking ($z = -1.6$, NS) than own urine, suggesting that urine of the same MUP type was not distinguished from own urine, despite many other genetic differences between brothers. Brother's urine of different MUP type stimulated significantly more investigation ($t_7 = -3.25$, $P < 0.01$) and scent marking ($z = 1.84$, $P < 0.05$) than that from a brother of the same MUP type as the subject, suggesting that urinary MUP type is an important factor in recognition of individual scent donors.

Brothers were previously familiar to the test animal (although animals were separated at least four weeks before testing) and unrelated stimulus donors were unfamiliar. However, there was no difference in investigation ($t_7 = 0.28$, NS) or scent marking ($z = -0.53$, NS) in response to urine of different MUP type, whether from an unrelated male or a brother (Fig. 2). Males respond to any competing scents from other males within their territories, whether these come from a familiar relative or an unfamiliar unrelated male²³. Thus, responses were not due to differences in familiarity. The MUP types of brothers often differed by only a few MUP bands (Fig. 1), but this still stimulated a strong response. The entire pattern of MUPs expressed thus appears to be used to discriminate own urine from that of another male.

To show that MUPs themselves were responsible for the response, we modified natural urine samples with recombinant MUP (rMUP), expressed in the yeast *Pichia pastoris*, the structure of which has been shown to resemble closely that of the protein from urine²⁴. Mice were presented with own urine or with own urine to which rMUP had been added (Fig. 3). Although own urine mixed with rMUP did not induce any more investigation than own urine alone ($z = -0.49$, NS), the addition of rMUP stimulated significantly more scent-marking activity ($z = -2.045$, $P < 0.025$, Fig. 4). As in previous tests, marking increased on both the stimulus and water-marked squares presented simultaneously.

The response to urine supplemented with rMUP confirms that mice can perceive differences in urinary MUP type. The characteristics of MUPs—genome derived, a very high level of individual heterogeneity, stable expression patterns, involatility, expression in large quantities and resistance to degradation—are all particularly suited to a role in communication of individual identity in scent marks. This might be true for both sexes, since wild-caught female mice also excrete substantial quantities of MUPs with similar heterogeneity²². Indeed, to our knowledge, no other role for MUPs expressed by female mice has been postulated. Further, MUPs are expressed by all adult males, regardless of status. Social status among male mice is signalled through levels of sesquiterpenes

excreted into the urine from preputial glands²⁵. However, scent signals deposited in the environment also need to provide information on the identity of the signaller. Females discriminate between competing males according to which male's marks were more freshly deposited (that is, the counter-marking male and winner of a competitive encounter), so unambiguous information on the identity of a scent mark owner is also crucial for mate selection¹⁹. It is not clear whether mice detect urinary MUP type through differences in volatile ligands or in the MUPs themselves. Volatile ligands released from MUPs attract mice to investigate urine scent marks, leading to direct contact¹⁰ and therefore bringing the involatile proteins into direct contact with receptors in the vomeronasal organ similar to those of rats that bind $\alpha 2u$ -globulins²⁶, the equivalent lipocalins in rat urine. MUPs stripped of their volatile ligands elicit immediate early gene *egr-1* expression in mitral cells of both the anterior and posterior accessory olfactory bulb in mice and appear to convey the strain recognition signals of the male pheromone responsible for pregnancy block¹⁷. Although most polymorphism occurs on the surface of the protein, some variants occur in the cavity-binding site and we have shown that these variants are able to modulate binding of ligands²⁷. Thus, MUP type may also affect volatile urinary odours through differential binding and release of volatile odorants. In this context, it is particularly interesting that noticeable differences in MHC-based odours among MHC-congenic inbred strains involve volatiles bound and released by urinary proteins^{28,29}. It has been assumed that these odours derive from volatiles bound to MHC proteins or their degradation products³⁰, but a role for MUPs, which have evolved to bind lipophilic molecules, has not been examined. MUP type and MHC haplotype, derived from gene clusters on different chromosomes and inherited independently, may combine to provide a highly polymorphic individual identity signal that is unlikely to be shared even between relatives. However, in this study of wild mice, using a behavioural response that reflects natural behaviour, there was no significant response to brothers of the same MUP type, even though these males were likely to be of different MHC type to the subjects. Thus, MUPs may constitute a significant part of the individuality signal, although the interplay between the two systems, and the precise mechanisms of chemical mediation, are yet to be resolved. □

Methods

In the first set of tests, adult male house mice (F_1 or F_2 outbred crosses of mice captured from geographically separate locations) were housed in separate laboratory enclosures (1.2×1.2 m)¹⁰ and presented with different urine stimuli (own, unrelated unfamiliar male, male sibling of different MUP type to own and male sibling of same MUP type as own) and a control test (two water stimuli) in a balanced order at weekly intervals. In each test, $2 \times 5 \mu\text{l}$ urine and $2 \times 5 \mu\text{l}$ water were streaked in the centres of separate 15×15 cm Perspex tiles covered in absorbent paper (Benchkote) and placed against opposite walls of a male's home enclosure. Investigatory behaviour (time in contact with each tile) was video recorded for the first 30 min of each test. Tiles were removed after 21 h and the number of marks counted under an ultraviolet lamp (marks smaller than 1×1 mm were not counted to exclude footprints). To stimulate competitive scent-marking behaviour¹⁰, a mesh grille (5 cm diameter) provided olfactory contact between pairs of neighbour unrelated males before and between tests. In addition, nest material from caged females was introduced for 20 h immediately before each test. During tests, grilles were covered and female odours removed. Because subordinate or non-competitive males suppress scent marking and do not respond to the urine of other males by increasing their scent-marking rates¹⁸, we first screened males for their willingness to scent-mark a tile streaked with unrelated male urine. Nine out of 18 males tested deposited very few scent marks (<20) and were excluded from further analyses¹⁰.

In the second set of tests, adult male subjects were tested as above with their own urine and independently with a sample of their own urine modified by addition of MUP to a level of 20% of the total protein. Recombinant MUP was prepared by heterologous expression in *Pichia pastoris*²⁴ and was purified by a four-step process, including two stages of high-resolution anion exchange chromatography. In some individuals, the rMUP comigrated with existing MUPs, although this cannot be taken as proof that they were the same protein²¹. In others, new bands were added to the MUP profile. The urine (own or mixed with rMUP, Fig. 3) was allowed to stand for 30 min (to allow rMUP to equilibrate with the semiochemical pool in the urine) before an amount of protein equivalent to that in $10 \mu\text{l}$ of own urine (range $40\text{--}130 \mu\text{g}$ protein) was deposited on the test substrate. In addition, $2 \times 5 \mu\text{l}$ water was deposited on a second piece of Benchkote and placed against

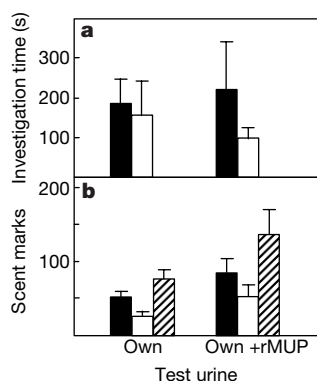


Figure 4 The effect of recombinant MUP on investigation and counter-marking of urine marks. Investigation (**a**) and scent marking (**b**) by adult male wild house mice presented with their own urine or their own urine mixed with recombinant MUP (means $\pm$ s.e.m.) was assessed in two separate tests. In each test, mice were presented with both a urine (filled bars) and water (open bars) stimulus, as described in the Methods. Cross-hatched bars show scent marks totalled for each test.

the opposite wall of the male's home enclosure. Investigation and scent marking were measured as described above. Tests were conducted four days apart in balanced order. Before tests, a female was introduced into each male's enclosure for 24 h, the female was then removed and males in neighbour enclosures were allowed a single interaction to stimulate more males to show competitive scent marking. Eleven out of 15 subjects deposited at least 20 marks on each tile and were included in analysis.

Received 16 August; accepted 17 September 2001.

1. Brown, R. E. & McDonald, D. W. *Social Odours in Mammals* Vols 1 & 2 (Clarendon, Oxford, 1985).
2. Yamazaki, K., Singer, A. & Beauchamp, G. K. Origin, functions and chemistry of H-2 regulated odorants. *Genetica* **104**, 235–240 (1999).
3. Yamazaki, K. *et al.* Sensory distinction between H-2b and H-2bm1 mutant mice. *Proc. Natl Acad. Sci. USA* **80**, 5685–5688 (1983).
4. Singh, P. B., Brown, R. E. & Roser, B. MHC antigens in urine as olfactory recognition cues. *Nature* **327**, 161–146 (1987).
5. Beynon, R. J., Robertson, D. H. L., Hubbard, S. J., Gaskell, S. J. & Hurst, J. L. in *Advances in Chemical Communication in Vertebrates* (eds Johnston, R. E., Muller-Schwarze, D. & Sorensen, P.) 137–147 (Plenum, New York, 1999).
6. Bacchini, A., Gaetani, E. & Cavaggoni, A. Pheromone binding proteins of the mouse, *Mus musculus*. *Experientia* **48**, 419–421 (1992).
7. Robertson, D. H. L., Beynon, R. J. & Evershed, R. P. Extraction, characterization and binding analysis of two pheromonally active ligands associated with major urinary protein of house mouse (*Mus musculus*). *J. Chem. Ecol.* **19**, 1405–1416 (1993).
8. Beynon, R. J. *et al.* in *Chemical Signals in Vertebrates* (eds Marchelewska-Koj, A., Muller-Schwarze, D. & Lepri, J.) 149–156 (Plenum, New York, 2001).
9. Hurst, J. L., Robertson, D. H. L., Tolladay, U. & Beynon, R. J. Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. *Anim. Behav.* **55**, 1289–1297 (1998).
10. Humphries, R. E., Robertson, D. H. L., Beynon, R. J. & Hurst, J. L. Unravelling the chemical basis of competitive scent marking in house mice. *Anim. Behav.* **58**, 1177–1190 (1999).
11. Mucignat-Caretta, C. & Caretta, A. Urinary chemical cues affect light avoidance behaviour in male laboratory mice, *Mus musculus*. *Anim. Behav.* **57**, 765–769 (1999).
12. Jemiolo, D., Alberts, J., Sochinski-Wiggins, S., Harvey, S. & Novotny, M. Behavioural and endocrine responses of female mice to synthetic analogs of volatile compounds in male urine. *Anim. Behav.* **33**, 1114–1118 (1985).
13. Novotny, M. V., Ma, W., Wiesler, D. & Zidek, L. Positive identification of the puberty-accelerating pheromone of the house mouse: the volatile ligands associating with the major urinary protein. *Proc. R. Soc. Lond. B* **266**, 2017–2022 (1999).
14. Jemiolo, B., Harvey, S. & Novotny, M. Promotion of the Whitten effect in female mice by synthetic analogs of male urinary constituents. *Proc. Natl Acad. Sci. USA* **83**, 4576–4579 (1986).
15. Marchelewska-Koj, A., Cavaggoni, A., Mucignat-Caretta, C. & Olejniczak, P. Stimulation of estrus in female mice by male urinary proteins. *J. Chem. Ecol.* **26**, 2355–2366 (2000).
16. Novotny, M., Harvey, S., Jemiolo, B. & Alberts, J. Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl Acad. Sci. USA* **82**, 2059–2061 (1985).
17. Brennan, P. A., Schellinck, H. M. & Keverne, E. B. Patterns of expression of the immediate-early gene *egr-1* in the accessory olfactory bulb of female mice exposed to pheromonal constituents of male urine. *Neuroscience* **90**, 1463–1470 (1999).
18. Nevison, C. M., Barnard, C. J., Beynon, R. J. & Hurst, J. L. The consequences of inbreeding for recognising competitors. *Proc. R. Soc. Lond. B* **267**, 687–694 (2000).
19. Hurst, J. L. *et al.* in *Chemical Signals in Vertebrates* (eds Marchelewska-Koj, A., Muller-Schwarze, D. & Lepri, J.) 43–50 (Plenum, New York, 2001).
20. Bishop, J. O., Clark, A. J., Clissold, P. M., Hainey, S. & Francke, U. Two main groups of mouse major urinary protein genes, both largely located on chromosome 4. *EMBO J.* **1**, 615–620 (1982).
21. Robertson, D. H., Cox, K. A., Gaskell, S. J., Evershed, R. P. & Beynon, R. J. Molecular heterogeneity in the major urinary proteins of the house mouse *Mus musculus*. *Biochem. J.* **316**, 265–272 (1996).
22. Payne, C. E. *et al.* in *Chemical Signals in Vertebrates* (eds Marchelewska-Koj, A., Muller-Schwarze, D. & Lepri, J.) 233–240 (Plenum, New York, 2001).
23. Hurst, J. L. Urine marking in populations of wild house mice *Mus domesticus* Rutt. 1. Communication between males. *Anim. Behav.* **40**, 209–222 (1990).
24. Lücke, C. *et al.* Solution structure of a recombinant mouse major urinary protein. *Eur. J. Biochem.* **266**, 1210–1218 (1999).
25. Novotny, M., Harvey, S. & Jemiolo, B. Chemistry of male dominance in the house mouse, *Mus domesticus*. *Experientia* **46**, 109–113 (1990).
26. Krieger, J. *et al.* Selective activation of G protein subtypes in the vomeronasal organ upon stimulation with urine-derived compounds. *J. Biol. Chem.* **274**, 4655–4662 (1999).
27. Marie, A. D. *et al.* Effect of polymorphisms on ligand binding by mouse major urinary proteins. *Protein Sci.* **10**, 411–417 (2001).
28. Singer, A. G., Tsuchiya, H., Wellington, J. L., Beauchamp, G. K. & Yamazaki, K. Chemistry of odortypes in mice—fractionation and bioassay. *J. Chem. Ecol.* **19**, 569–579 (1993).
29. Singer, A. G., Beauchamp, G. K. & Yamazaki, K. Volatile signals of the major histocompatibility complex in male mouse urine. *Proc. Natl Acad. Sci. USA* **94**, 2210–2214 (1997).
30. Pearce-Pratt, R., Schellinck, H., Brown, R., Singh, P. B. & Roser, B. Soluble MHC antigens and olfactory recognition of genetic individuality: the mechanism. *Genetica* **104**, 223–230 (1999).

Acknowledgements

This work was supported by Biotechnology and Biological Sciences Research Council (BBSRC) grants to J.L.H. and R.J.B., a BBSRC studentship to C.E.P. and an Egyptian government scholarship to A.D.M.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to J.L.H. (e-mail: jane.hurst@liverpool.ac.uk).

Drosophila Stardust interacts with Crumbs to control polarity of epithelia but not neuroblasts

Yang Hong*, Beth Stronach†, Norbert Perrimon†, Lily Yeh Jan* & Yuh Nung Jan*

* Howard Hughes Medical Institute, Department of Physiology and Biochemistry, University of California San Francisco, San Francisco, California 94143-0725, USA

† Department of Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115, USA

Establishing cellular polarity is critical for tissue organization and function. Initially discovered in the landmark genetic screen for *Drosophila* developmental mutants^{1–4}, *bazooka*, *crumbs*, *shotgun* and *stardust* mutants exhibit severe disruption in apicobasal polarity in embryonic epithelia, resulting in multilayered epithelia, tissue disintegration, and defects in cuticle formation⁵. Here we report that *stardust* encodes single PDZ domain MAGUK (membrane-associated guanylate kinase) proteins that are expressed in all primary embryonic epithelia from the onset of gastrulation. *Stardust* colocalizes with *Crumbs*⁶ at the apicolateral boundary, although their expression patterns in sensory organs differ. *Stardust* binds to the carboxy terminus of *Crumbs* *in vitro*, and *Stardust* and *Crumbs* are mutually dependent in their stability, localization and function in controlling the apicobasal polarity of epithelial cells. However, for the subset of ectodermal cells that delaminate and form neuroblasts, their polarity requires the function of *Bazooka*^{7,8}, but not of *Stardust* or *Crumbs*.

The *stardust* (*sdt*) mutation is not complemented by *Df(1)HA11*, a deletion of regions 7D14–7D22 (ref. 9). *HA11* was mapped to a region of about 85 kilobases (kb) (B.S., unpublished data), predicted to contain six open reading frames of more than 300 amino acids each by the genome annotation database of *Drosophila* (GadFly, <http://www.bdgp.org>). One of these open reading frames, CG1617, encodes a previously unknown MAGUK protein containing a single PDZ (PSD-95, Discs Large, ZO-1) domain, a SH3 (Src homology region 3) domain and a GUK (guanylate kinase) domain. We pursued the possibility that this MAGUK protein corresponds to *Sdt*, because other proteins with similar motifs are important for cell–cell junctions and cellular polarity^{10–14}.

To obtain full-length complementary DNAs, we screened an embryonic cDNA library and identified a large transcription unit that includes CG1617 and CG15341. Three cDNAs for this *sdt* candidate gene, *sdt1*, *sdt2* and *sdt3* (Fig. 1a), differ at their 5' ends owing to alternative splicing, and code for two isoforms of potential *Sdt* protein: *SdtA*, with 1,292 amino acids; and *SdtB*, with 860 amino acids and lacking the 432 amino acids encoded by alternatively spliced exon 3 (hatched bar in Fig. 1d). *In vitro* translation of *sdt1* and *sdt3* yielded products of the predicted size (Fig. 1b). Blast analyses (<http://www.ncbi.nlm.nih.gov/BLAST>) identified homologues of *SdtB* in mouse, recently identified as Pals1 (proteins associated with mammalian Lin-7 (ref. 15), and in *Caenorhabditis elegans* a predicted protein of unknown function (see Supplementary Information). No homology to the amino-acid sequence of exon 3 in *SdtA* was found.

The gene that gives rise to these three cDNAs is *sdt*, because three independent strong hypomorphic or null alleles of *sdt*—*XN05*, *XP96* and *EH*—induced by ethylmethane sulphonate (EMS)^{9,16} each carry a single nucleotide alteration in the coding sequences for *sdt1*–3. *XN05* contains a nonsense mutation in exon 6 (Fig. 1d). *XP96* contains a mutation at the 3' splice junction of exon 6; a failed splicing would incorporate a stop codon that immediately follows

this splice junction. *EH* contains a nonsense mutation in exon 3, which should eliminate SdtA but not SdtB, indicating that SdtA serves a critical function in embryogenesis.

Affinity-purified rabbit antibodies against a glutathione S-transferase (GST) fusion of the C-terminal 200 amino acids common to

SdtA and SdtB clearly outlined epithelial cells in wild-type embryos (Fig. 1c, right), but not in zygotic *XN05* (Fig. 1c, left) or *XP96* mutant embryos (data not shown). The speckles seen in mutant as well as wild-type embryos are background nonspecific staining. From the onset of gastrulation (stage 5 or 6), Sdt is expressed

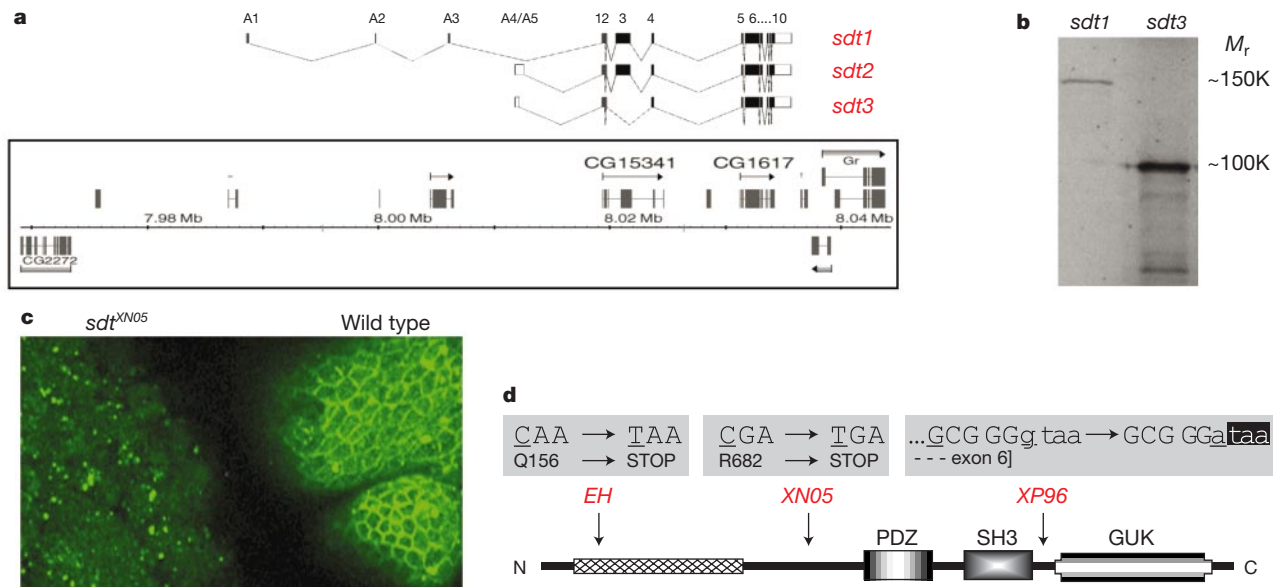


Figure 1 Cloning of *sdt*. **a**, Three alternatively spliced cDNA isoforms of *sdt*. Upstream noncoding exons are designated as A1–A5, and the exon that includes the first in-frame ATG is designated as exon 1. Boxed is a modified GadFly screen display of the 7D18 region of the X chromosome, with arrows indicating the direction of transcription, and bars indicating predicted exons. **b**, *In vitro* translation products of *sdt1* and *sdt3* cDNAs. Each cDNA yields a major protein product of the expected size: relative molecular mass (M_r)

~150,000 (150K) for SdtA and ~100K for SdtB. **c**, Sdt antibody outlines epithelial cells in wild-type but not *sdt^{XN05}* zygotic mutant embryo. Both embryos are at about stage 11. The speckles shown in the *XN05* embryo are nonspecific background staining. **d**, SdtA protein structure and lesions in three *sdt* alleles. Hatched bar represents the amino-acid sequence of the alternatively spliced exon 3.

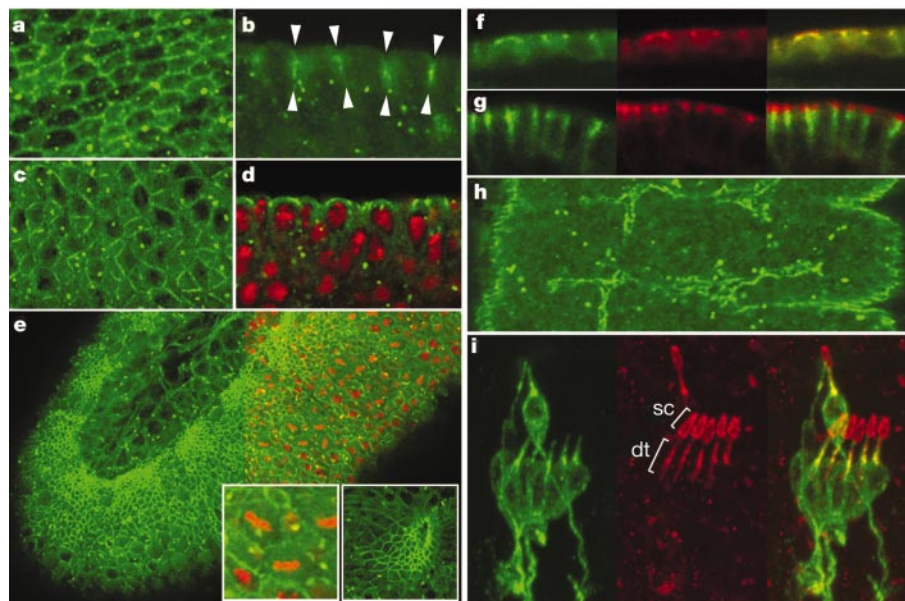


Figure 2 Developmental expression pattern of Sdt. **a**, **b**, Sdt is first detectable around stage 5–6, and is enriched at the boundary between epithelial cells, seen in tangential section (**a**) and cross section (**b**) of embryos. Vertical pairs of arrowheads in **b** highlight the Sdt distribution along the apicolateral cortex. **c**, **d**, At stage 8–9 Sdt concentrates around apical cell boundaries as seen in tangential section (**c**) and cross section (**d**) of the same embryo, with DNA stained by propidium iodide (PI) (red) in **d**. **e**, A honeycomb pattern of Sdt forms at stage 9. The right half is superimposed with DNA staining (red). The left inset shows that in cells undergoing mitosis, Sdt staining is diffuse or absent from the cell boundary. The right inset shows that Sdt is further enriched at the sites of contraction of

the apical cell surface during cell invagination (a tracheal pit is shown here). **f**, **g**, Cross section of a stage 13 (**f**) and a stage 14 (**g**) embryo. Sdt (red) overlaps with AJ marker Arm (green, **f**) but extends further apically. Sdt is apical to the septate junction marker Cor (green, **g**) with no overlap. **h**, Sdt expression at the apical lumen surface of the developing tracheal system in a stage 13 embryo. Anterior is up and dorsal left. **i**, Chordotonal organs double stained with Sdt antibody (red) and 22C10 (green), which specifically stains neurons of the peripheral and central nervous systems. Sdt is localized to the dendritic tips (dt) and scolopales (sc). In **f**, **g** and **i**, right panels are merged images.

around the apical cell boundaries (Fig. 2a) and the apical region of the lateral cortex (Fig. 2b). In late gastrulation, coincident with formation of the zonula adherens (ZA), Sdt starts to form a solid continuous belt around the apical boundary of epithelial cells, and is also enriched at the apical surface (Fig. 2c–e). Sdt distribution overlaps with adherens junction markers Armadillo (Arm) and DE-cadherin (DE-cad), although Sdt seems to localize slightly apical to adherens junctions (Fig. 2f) as well. When septate junctions form at the basolateral side of epithelial cells (embryonic stage 13 or 14), Sdt retains its apical localization and association with the ZA, and does not overlap with the septate junction marker Coracle (Cor) (Fig. 2g). The tracheal system, foregut and hindgut, which are part of the primary epithelia¹⁷, also express Sdt at their apical surface (Fig. 2h). Apical lateral subcellular distribution of Sdt persists throughout embryonic development in epithelia, although the expression gradually decrease in stages 16 and 17. Sdt is also expressed in sensory organs. For example, in antennomaxillary complexes and chordotonal organs, strong Sdt staining is seen on the dendritic tips of sensory neurons and scolopale lumen formed by support scolopale cells (Fig. 2i).

Adherens junctions (AJs) first form during cellularization (stage 5) in fly embryos as spot AJs^{17,18}, which later migrate apically and coalesce to form the ZA, a continuous belt-like AJ around the apical lateral cell boundary¹⁹. The *sdt* mutations seem to specifically disrupt the fusion of apically localized spot AJs to form the ZA^{19,20}. In *sdt* zygotic mutants, AJ/ZA components Arm and DE-cad fail to form a smooth honeycomb pattern, but appear significantly fragmented^{16,19,20} (see Fig. 3j, k; and data not shown). This failure of ZA formation and apicobasal polarity establishment results in an irregular and multilayered epithelial structure in later embryogenesis⁹ (Fig. 3b). Despite their initial normal distribution during gastrulation (stage 7 or 8), basolateral localization of Discs Large (Dlg) and Scribble (Scrib), which marks the future septate junctions, is progressively compromised in later stages (Fig. 3b, e). Another indication that septate junctions fail to develop in *sdt* mutants was the cytoplasmic distribution of the septate junction

Table 1 Localization of cellular polarity markers in stage 9–13 embryos

Protein	Localization in wild-type embryos		Localization in <i>sdt</i> embryos		Reduction of protein level in <i>sdt</i> mutant
	Epithelia	Neuroblast	Epithelia	Neuroblast	
Arm (Cyto)	A/J	–	A*, BL	–	Mild
DE-cad (TM)	A/J	–	A*, BL	–	Mild
Scrib (Cyto)	BL	–	C	–	Mild
Dlg (Cyto)	BL	–	C	–	Mild
Cor (Cyto)	BL	–	C	–	Mild
Baz (Cyto)	A	A	A*	A	Mild
aPKC (Cyto)	A	A	A*	A	Mild
DmPar-6 (Cyto)	A	A	A*	A	Mild
Mir (Cyto)	C	B	C	B	Mild
Crab (TM)	A	–	A*	–	Strong
Dlt (Cyto)	A	–	A*	–	Strong

Cyto, cytoplasmic; TM, transmembrane; A, apical; B, basal; BL, basolateral; J, adherens junctional; C, cortical.

*Punctate and fragmented staining around the cell boundary.

marker Cor at stage 14 (Fig. 3f). Table 1 summarizes the expression patterns of a battery of cellular polarity markers we have examined in *sdt* mutants, showing that all apicolateral and basolateral markers tested are mislocalized in *sdt* mutant epithelia.

Bazooka (Baz), DmPar-6 and atypical protein kinase C (aPKC)²¹ form a complex essential for both neuronal and epithelial polarity, and resemble Sdt in their epithelial expression and subcellular localization (Fig. 3a, c, g). As early as stage 7 these proteins all become mislocalized and form random aggregates around the apical cell boundary in epithelial cells (Fig. 3b, d, g, and data not shown). However, their apical localization in delaminating and dividing neuroblasts remains largely normal not only in zygotic *sdt* mutant embryos but also in germline clone embryos in which both maternal and zygotic *sdt* contributions are removed (Fig. 3h and data not shown). Likewise, the basal localization of Miranda (Mir)²² in neuroblasts was unaffected in *sdt* zygotic or germline clone embryos (Fig. 3i). Similar results were also seen in zygotic *crb* mutants (data not shown), indicating that epithelial but not

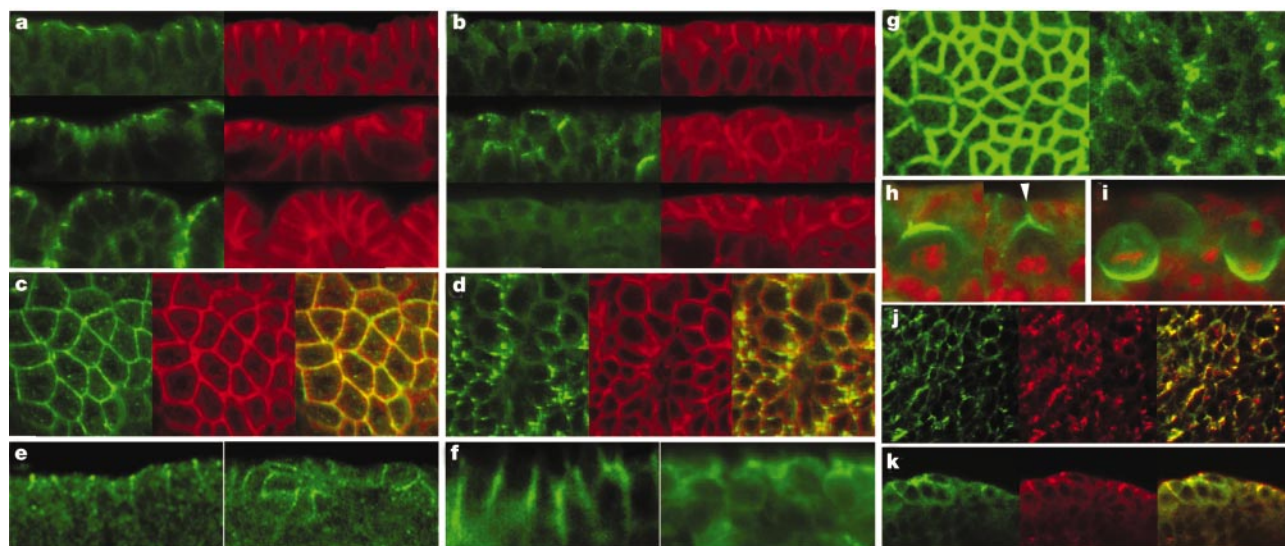


Figure 3 Characterization of polarity phenotype in *sdt* mutants. **a–d**, Epithelial expression and localization of Baz (green) and Dlg (red) in wild-type (**a, c**) and *XP96* germline clone embryos (**b, d**), shown in cross section at stages 7–8, 11–12 and 14 (**a, b**, from top to bottom), respectively, and in tangential section at stage 9 (**c, d**; right panels are merged images). Note the irregular cell shape and multilayered epithelial structure revealed by Dlg staining in *XP96* mutants at stages 11–12 and 14. **e**, Scrib localization in stage 10–11 wild-type (left) and *XN05* mutant (right) epithelia are very similar to that of Dlg. **f**, Cor is localized to septate junctions in the wild type (left), but appears to be mislocalized into cytoplasm in *XN05* mutant (right). Embryos are at stage 14. **g**, Similar to Baz, aPKC is

uniformly localized to the apical cell cortex of wild-type epithelia (left), but is mislocalized into random aggregates around apical cell boundaries in *XN05* mutant embryos (right). Embryos in **g–k** are all at stage 10. **h, i**, Apical crescent of Baz (**h**) and basal crescent of Mir (**i**) in neuroblasts are both unaffected in *XP96* germline clone embryos that lack both zygotic and maternal *sdt* products. Arrowhead in **h** highlights a Baz ‘stalk’ expanding into the crescent. In **h** and **i**, DNA is stained with PI (red). **j, k**, Although Arm (green) and Baz (red) are mislocalized in *sdt* mutant embryos, they remain largely colocalized around apical cell boundaries (**j**, tangential view, *XP96* germline clone embryo; **k**, cross section view, *XN05* mutant embryo). Right panels are merged images.

neuroblast polarity requires Sdt and Crb.

We wondered how neuroblasts retained their normal apicobasal polarity in *sdt* mutants. Unlike Crb and Dlt (see below), the expression levels of Baz, DmPar-6 and aPKC are not significantly reduced in *sdt* mutants before stage 13 (Fig. 3a–d). Moreover, Baz remains colocalized with Arm/DE-cad in the disrupted AJs (Fig. 3j, k), indicating a residual apicobasal polarity in *sdt* mutants. This is probably due to partial redundancy between Baz and Sdt, as removing zygotic copies of *baz* significantly enhances the null phenotype of *sdt*¹⁶. This residual polarity retains Baz apically in *sdt* mutants, so that as a neuroblast delaminates Baz is still concentrated in the apical stalk structure^{7,8} that expands into an apical crescent (Fig. 3h).

Crumb, a transmembrane protein with EGF-like repeats, is an apical determinant for establishing apicobasal polarity in embryonic epithelia^{6,23,24}. The *sdt* and *crb* mutants exhibit nearly identical defects in epithelial apicobasal polarity, and *crb* and *sdt* function in a common genetic pathway^{9,19,20}. We found precise colocalization of Sdt and Crb throughout embryonic development in all cells examined (Fig. 4a–d) except for those in sensory organs: Sdt but not Crb is at the distal dendrites of sensory neurons (Fig. 4e). In epithelial cells Crb and Sdt are mutually dependent for their localization and stability. There is a marked reduction of the protein levels of Crb in *sdt* mutants⁹ and of Sdt in *crb* mutants after gastrulation. In both cases, instead of the normal apicolateral distribution, these proteins are found in sparse and discrete random spots (Fig. 4f and g). Dlt, which interacts with the Crb intracellular domain, is distributed similarly to Crb in *sdt* mutants (Fig. 4h), although its early expression and localization during cellularization is not affected even in *sdt* germline clone embryos (data not shown). Conversely, overexpression of the Crb intra-

cellular domain (Crb-intra) is sufficient to cause a 'dominant' phenotype, disrupting cell polarity, expanding the apical domain, and displacing endogenous Crb into cytoplasm^{23,24}. In such embryos we find that a large fraction of Sdt, together with Crb and Crb-intra, is also displaced away from the membrane into the cytoplasm (Fig. 4i).

To test whether the mutual dependence of Sdt and Crb is due to their physical interaction, we carried out a GST::Crb-intra pull-down assay using *in vitro* translated ³⁵S-labelled Sdt. Both SdtA and SdtB bind specifically to Crb-intra (Fig. 4k). Two domains of Crb-intra are known²³, the C-terminal domain containing the EERLI motif, and the amino-terminal domain containing amino acids, such as Y10 and E16, that are conserved in Crb and its *C. elegans* homologues²³ (Fig. 4j). Mutation in either domain abolishes the ability of Crb-intra to rescue the *crb* phenotype, but only the EERLI motif is required for inducing the dominant overexpression phenotype of Crb-intra²³. In the *in vitro* binding assay, binding of Sdt to Crb-intra was nearly abolished by removal of the C-terminal EERLI motif, but was not affected by Y10A and/or E16A mutations (Fig. 4k), indicating that Crb binds Sdt with its C-terminal EERLI motif.

The same C-terminal EERLI is also required for Dlt to bind to Crb²³. It will be of interest to determine whether Sdt and Dlt bind Crb cooperatively to form a tertiary complex, or whether they compete with each other for binding to the Crb intracellular domain. We did not detect any direct binding between Dlt and Sdt using the same *in vitro* GST pull-down assay (data not shown). However, MAGUK proteins frequently show intramolecular auto-inhibition between different binding motifs^{25,26} so it remains possible that Sdt binds Dlt only after it first binds to Crb.

Thus, whereas the Crb–Sdt (and possibly Dlt) pathway controls

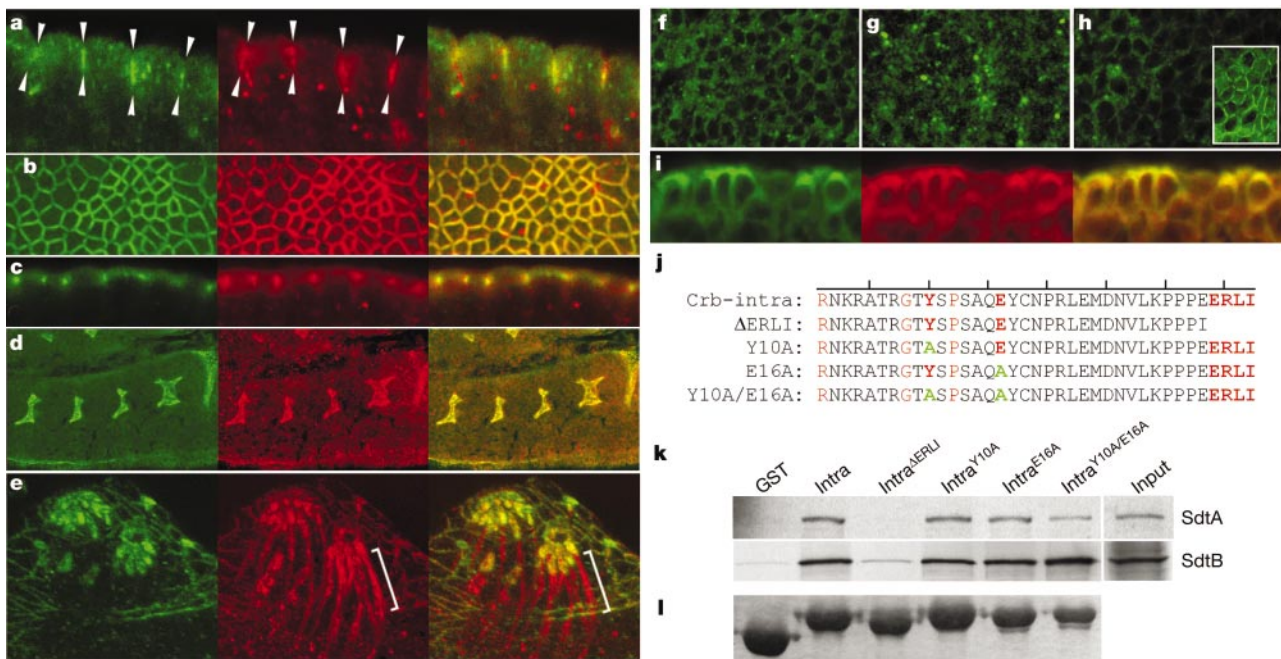


Figure 4 Sdt interacts with Crb. **a–d**, Colocalization of Crb (green) and Sdt (red) in embryonic epithelial cells. **a**, Cross section, stage 5–6; **b**, tangential view, stage 11; **c**, stage 9, cross section; **d**, stage 11, cross section, tracheal pits. In **a–e** and **i**, right panels are merged images. Vertical pairs of arrowheads in **a** highlight the Sdt and Crb distribution along the apicolateral cortex. **e**, Expression of Crb (green) and Sdt (red) in antennomaxillary complexes in stage 16 embryo. Here, Sdt but not Crb is expressed in the distal dendrites of the sensory neurons (brackets). **f**, Crb localization is severely disrupted and the protein level is reduced in stage 10 *sdt*^{KNO5} zygotic mutant epithelia. **g**, Sdt localization and expression in epithelia is similarly disrupted and reduced in stage 10 *crb*^{T1A22} zygotic mutants. **h**, Epithelial expression of Dlt in *sdt*^{KNO5} stage 10 mutant embryo

looks similar to Crb expression in **f**. Inset, Dlt expression in the wild type. **i**, Stage 9 embryo overexpressing Crb-intra shows redistribution and colocalization of endogenous Crb (green) and Sdt (red) to the cytoplasm and the expanded apical surface. **j**, Sequences of Crb intracellular domain and different mutants used in GST pull-down experiments (**k**). Conserved amino acids in Crb and its *C. elegans* homologues (not shown) are in red, and mutated amino acids are in green. Residues implicated in Crb function in mutagenesis studies²³ are in bold. **k**, *In vitro* GST pull-down assay demonstrating the interaction between Crb-intra and ³⁵S-labelled SdtA and SdtB. In the Input panel, only half the amount of the ³⁵S-labelled SdtA and SdtB proteins were loaded owing to the limitation of loading volume. **l**, SDS-PAGE gel showing the relative amount of protein used in assays in **k**.

the epithelial but not neuroblast polarity, the Baz–DmPar-6–aPKC complex affects both epithelial and neuroblast polarity. Proteins in both pathways are expressed in epithelial cells with identical apico-lateral localization patterns^{7,8,12,21} (data not shown) but only Baz–DmPar-6–aPKC are expressed in neuroblasts. In epithelia, these two pathways are probably partially redundant in controlling apicobasal polarity. Their role in regulating ZA formation seems to be the key to their epithelial and cuticle phenotypes. Sdt and Crb colocalize in epithelia and together serve as apical determinants, but only partially overlap and are likely to serve different functions in sensory organs. A mutation in a human Crb homologue has been implicated as the cause of one form of retinitis pigmentosa²⁷, suggesting a potential role for the Crb family of proteins in the localization of a sensory transduction complex²⁸. The localization of Sdt to the dendritic tip and scolopale cells, the probable sites for sensory transduction, raises the possibility that Sdt may have a role in the development or function of a sensory transduction apparatus. □

Methods

Fly stocks and molecular biology

y w was used as the wild-type stock in all experiments. For mutant phenotype analysis, *sdt*^{XN05} and *sdt*^{XP96} were balanced over *FM7, P{fz/lacZ}* and *crb*^{1A22} was balanced over *TM3, P{w⁺tmC = 35UZ} Sb¹. sdt^{XP96}* germline clone embryos were produced using the FLP/DFS technique as described¹⁶. Overexpression of Crb-intra was achieved by crossing upstream activating sequence (UAS)-Crb-intra²³ with the maternal GAL4 driver V32A (provided by D. St Johnston) at 25 °C. Crb-intra fragments were amplified by PCR and inserted into pGEX-4T-1 vector between *EcoRI* and *SalI* to make GST fusion constructs. The GST pull-down assay was carried out as described²².

Allele sequencing

Each *sdt* allele was rebalanced with a green fluorescent protein (GFP) X-chromosome balancer²⁹. About 40 GFP-deficient late-stage embryos were picked for DNA preparation. Genomic DNA fragments of *sdt* mutants were amplified with Roche Expand 20kb^{PLUS} PCR kit from two embryo equivalents of DNA (pre-denatured before PCR). PCR reactions were set up in duplicate or triplicate and each PCR fragment was independently cloned into pGEM-T Easy+ vector and both strands were fully sequenced.

Immunofluorescence staining of embryos

Rabbit anti-Sdt serum was affinity purified using Bio-Rad Affi-Gel bound with the same GST:Sdt C-terminal fusion protein used for immunization. Both crude and purified Sdt antiserum failed to detect specific signals in immunoblot experiments. Embryos were fixed with either 4% formaldehyde or 4% paraformaldehyde in 1× PBS buffer. Dilution factors for primary antibodies are given in Supplementary Information. Images were taken on a Bio-Rad MRC600 and a Bio-Rad 1024MP confocal microscope and processed with Adobe Photoshop software.

Received 5 June; accepted 24 September 2001.

- Nüsslein-Volhard, C. & Wieschaus, E. Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**, 795–801 (1980).
- Jurgens, G., Wieschaus, E., Nüsslein-Volhard, C. & Kluding, H. Mutation affecting the pattern of the larval cuticle in *Drosophila melanogaster*: II. Zygotic loci on the third chromosome. *Roux Arch. Dev. Biol.* **193**, 283–295 (1984).
- Nüsslein-Volhard, C., Wieschaus, E. & Kluding, H. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*: I. Zygotic loci on the second chromosome. *Roux Arch. Dev. Biol.* **193**, 267–282 (1984).
- Wieschaus, E., Nüsslein-Volhard, C. & Jurgens, G. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*: III. Zygotic loci on the X chromosome and fourth chromosome. *Roux Arch. Dev. Biol.* **193**, 296–307 (1984).
- Müller, H.-A. J. Genetic control of epithelial cell polarity: lessons from *Drosophila*. *Dev. Dyn.* **218**, 52–67 (2000).
- Tepass, U., Theres, C. & Knust, E. *crumbs* encodes an EGF-like protein expressed on apical membranes of *Drosophila* epithelial cells and required for organization of epithelia. *Cell* **61**, 787–799 (1990).
- Schober, M., Schaefer, M. & Knoblich, J. A. Bazooka recruits Inscutable to orient asymmetric cell divisions in *Drosophila* neuroblasts. *Nature* **402**, 548–551 (1999).
- Wodarz, A., Ramrath, A., Kuchinke, U. & Knust, E. Bazooka provides an apical cue for Inscutable localization in *Drosophila* neuroblasts. *Nature* **402**, 544–547 (1999).
- Tepass, U. & Knust, E. *crumbs* and *stardust* act in a genetic pathway that controls the organization of epithelia in *Drosophila melanogaster*. *Dev. Biol.* **159**, 311–326 (1993).
- Dimitratos, S. D., Woods, D. F., Stathakis, D. G. & Bryant, P. J. Signalling pathways are focused at specialized regions of the plasma membrane by scaffolding proteins of the MAGUK family. *BioEssays* **21**, 912–921 (1999).
- Gonzalez-Mariscal, L., Betanzos, A. & Avila-Flores, A. MAGUK proteins: structure and role in the tight junction. *Cell Dev. Biol.* **11**, 315–324 (2000).
- Petronczki, M. & Knoblich, J. A. DmPar-6 directs epithelial polarity and asymmetric cell division of neuroblasts in *Drosophila*. *Nature Cell Biol.* **3**, 43–49 (2001).
- Bhat, M. *et al.* Discs Lost, a novel multi-PDZ domain protein establishes and maintains epithelial polarity. *Cell* **96**, 833–845 (1999).

- Bilder, D. & Perrimon, N. Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* **403**, 676–680 (2000).
- Kamberov, E. *et al.* Molecular cloning and characterization of Pals, proteins associated with mLin-7. *J. Biol. Chem.* **275**, 11425–11431 (2000).
- Müller, H.-A. J. & Wieschaus, E. *armadillo*, *bazooka*, and *stardust* are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in *Drosophila*. *J. Cell Biol.* **134**, 149–163 (1996).
- Tepass, U. Epithelial differentiation in *Drosophila*. *BioEssays* **19**, 673–682 (1997).
- Tepass, U. & Hartenstein, V. The development of cellular junctions in the *Drosophila* embryo. *Dev. Biol.* **161**, 563–596 (1994).
- Tepass, U. Crumbs, a component of the apical membrane, is required for zonula adherens formation in primary epithelia of *Drosophila*. *Dev. Biol.* **177**, 217–225 (1996).
- Grawe, F., Wodarz, A., Lee, B., Knust, E. & Skaer, H. The *Drosophila* genes *crumbs* and *stardust* are involved in the biogenesis of adherens junctions. *Development* **122**, 951–959 (1996).
- Wodarz, A., Ramrath, A., Grimm, A. & Knust, E. *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J. Cell Biol.* **150**, 1361–1374 (2000).
- Shen, C.-P., Jan, L. Y. & Jan, Y. N. Miranda is required for the asymmetric localization of Prospero during mitosis in *Drosophila*. *Cell* **90**, 449–458 (1997).
- Klebes, A. & Knust, E. A conserved motif in Crumbs is required for E-cadherin localisation and zonula adherens formation in *Drosophila*. *Curr. Biol.* **10**, 76–85 (2000).
- Wodarz, A., Hinz, U., Engelbert, M. & Knust, E. Expression of Crumbs confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. *Cell* **82**, 67–76 (1995).
- Brennan, J. E. *et al.* Localization of postsynaptic density-93 to dendritic microtubules and interaction with microtubule-associated protein 1A. *J. Neurosci.* **18**, 8805–8813 (1998).
- Shin, H., Hsueh, Y.-P., Yang, F.-C., Kim, E. & Sheng, M. An intramolecular interaction between Src homology 3 domain and guanylate kinase-like domain required for channel clustering by Post-synaptic Density-95/SAP90. *J. Neurosci.* **20**, 3580–3587 (2000).
- Den Hollander, A. I. *et al.* Mutations in a human homologue of *Drosophila crumbs* cause retinitis pigmentosa (RP12). *Nature Genet.* **23**, 217–221 (1999).
- Rashbass, P. & Skaer, H. Cell polarity: nailing Crumbs to the scaffold. *Curr. Biol.* **10**, R234–R236 (2000).
- Casso, D., Ramirez-Weber, F. & Kornberg, T. B. GFP-tagged balancer chromosomes for *Drosophila melanogaster*. *Mech. Dev.* **91**, 451–454 (2000).

Supplementary Information accompanies the paper on Nature's website (<http://www.nature.com>).

Acknowledgements

We are grateful to D. Bilder, M. Bhat, T. Uemura, P. J. Bryant, U. Tepass, E. Knust, E. Wieschaus, Developmental Studies Hybridoma Bank and Bloomington Stock Center for providing fly stocks, antibodies and constructs. We thank S. Barbel, L. Ackerman and S. Younger-Shepherd for technical assistance and M. Rothenberg, D. Cox, S. Abdelilah and U. Tepass for critical comments on the manuscript. B.S. is supported by a grant from the National Institutes of Health. Y.H. is a Research Associate and N.P., L.Y.J. and Y.N.J. are Investigators of the Howard Hughes Medical Institute.

Correspondence and requests for materials should be addressed to Y.N.J. (e-mail: ynjan@itsa.ucsf.edu).

Drosophila Stardust is a partner of Crumbs in the control of epithelial cell polarity

André Bachmann, Martina Schneider*, Eva Theilenberg, Ferdi Grawe & Elisabeth Knust

Institut für Genetik, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany

The polarized architecture of epithelial cells depends on the highly stereotypic distribution of cellular junctions and other membrane-associated protein complexes. In epithelial cells of the *Drosophila* embryo, three distinct domains subdivide the lateral plasma membrane. The most apical one comprises the subapical complex (SAC). It is followed by the zonula adherens (ZA) and, further basally, by the septate junction¹. A core component of the SAC is the transmembrane protein Crumbs, the cytoplasmic domain of which recruits the PDZ-protein Discs Lost into the complex^{2,3}. Cells lacking *crumbs* or the functionally related gene *stardust* fail to organize a continuous ZA and to maintain cell

* Present address: Lund University, Department of Cell & Molecular Biology, S-22184 Lund, Sweden.

polarity⁴⁻⁶. Here we show that *stardust* provides an essential component of the SAC. Stardust proteins colocalize with Crumbs and bind to the carboxy-terminal amino acids of its cytoplasmic tail. We introduce two different Stardust proteins here: one MAGUK protein, characterized by a PDZ domain, an SH3 domain and a guanylate kinase domain; and a second isoform comprising only the guanylate kinase domain. The Stardust proteins represent versatile candidates as structural and possibly regulatory constituents of the SAC, a crucial element in the control of epithelial cell polarity.

The allele *sdt*^{P10} was induced by mobilization of the P-element of the viable and fertile insertion line *Is(1)P[ry⁺]³³⁴*. *sdt*^{P10} exhibits an intermediate *sdt* phenotype and fails to complement other *sdt* alleles (Fig. 1a–c). Molecular cloning of flanking DNA allowed to map the P-element of *Is(1)P[ry⁺]³³⁴* about 20 kilobases (kb) distal to that of *sdt*^{P10} and very close to the enhancer-promoter (EP)-element of the homozygous viable EP-line *EP(X)1143* (Fig. 2a). Imprecise excision of the EP-element induced additional *sdt* alleles (data not shown).

We recovered more than ten related but incomplete complementary DNAs and used them to isolate additional cDNAs. The 5.7-kb

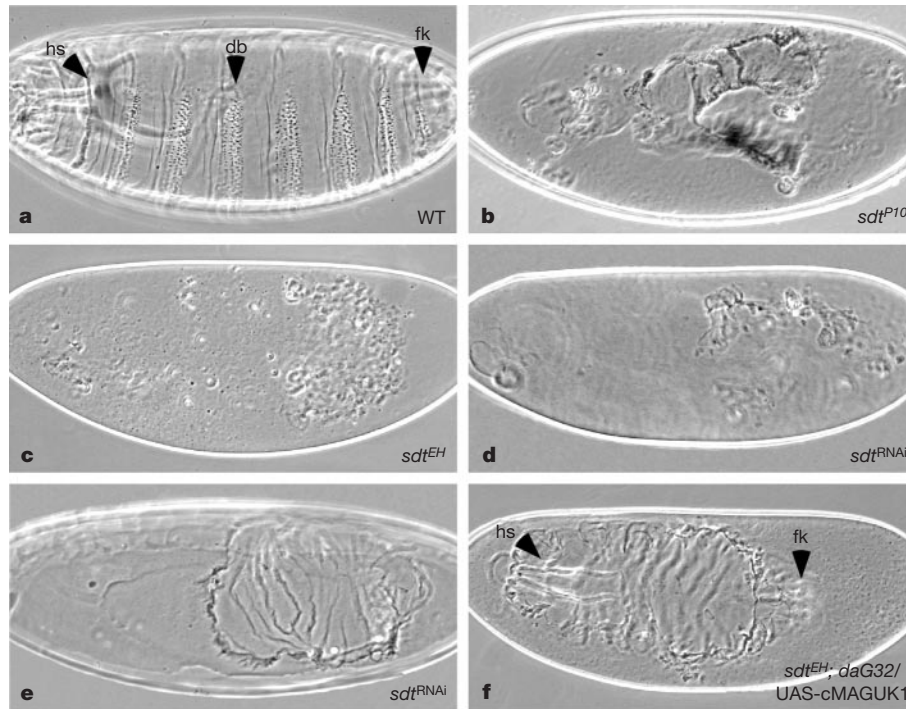


Figure 1 Cuticle preparations of embryos. **a**, Wild-type embryo. **b**, *sdt*^{P10}/Y embryo with an intermediate phenotype. **c**, *sdt*^{EH}/Y embryo with a strong phenotype. **d**, **e**, Wild-type embryos injected with MAGUK dsRNA (RNAi). Embryos show a variable expression level of the mutant phenotype (42% weak, 40% intermediate (**e**) and 18% strong (**d**) phenotypes).

The same classes of mutant phenotypes were achieved on injection of either SH3 dsRNA or N-term dsRNA. **f**, *sdt*^{EH}/Y; *daG32*/UAS-cMAGUK1 embryo. Large parts of continuous cuticle and the head skeleton are restored. hs, head skeleton; db, denticle belts; fk, filzkörper. Anterior is to the left.

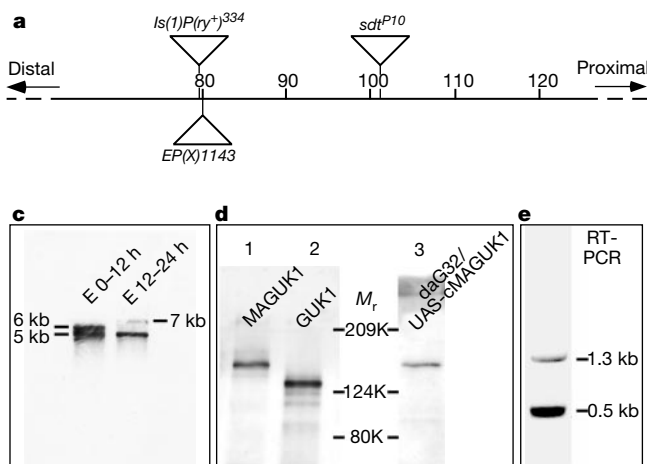
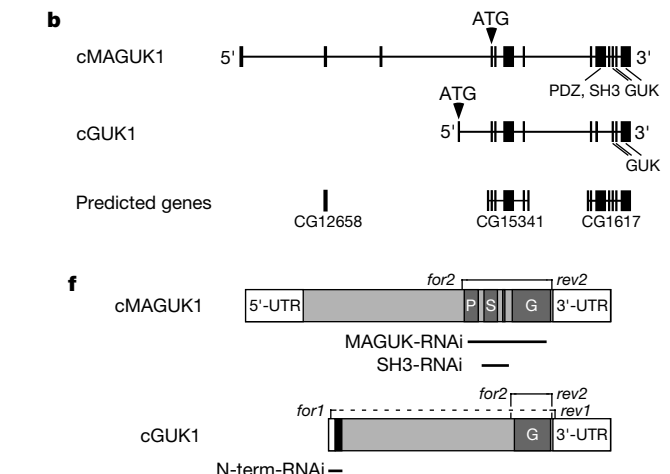


Figure 2 Molecular analysis of the *sdt* locus. **a**, Genomic region from the cytological interval 7D18. Triangles are P-element inserts. Numbering refers to genomic scaffold AE003443. **b**, cDNAs cMAGUK1 and cGUK1. Predicted genes refer to those assumed by the Berkeley Drosophila Genome Project. ATG, putative translation initiation codon. **c**, Northern blot of embryonic poly(A)⁺ RNA, hybridized with a probe covering the MAGUK domain. **d**, *In vitro* translated Sdt-MAGUK1 (lane 1) and Sdt-GUK1 (lane 2) and extracts of



embryos (*daG32*/UAS-cMAGUK1) (lane 3), detected with the anti-Sdt^{pep153} antiserum. **e**, Products of RT-PCR (see Methods). The amplified fragments correspond to those coding for the MAGUK (1.3 kb) and GUK (0.5 kb) domains, respectively. **f**, cMAGUK1 and cGUK1. Grey, ORF; black bar in cGUK1, cGUK1-specific N-terminal sequence (N-term-RNAi). P, PDZ domain; S, SH3 domain; G, GUK domain. Bold bars, regions used for RNAi; *for* and *rev*, primers used for RT-PCR.

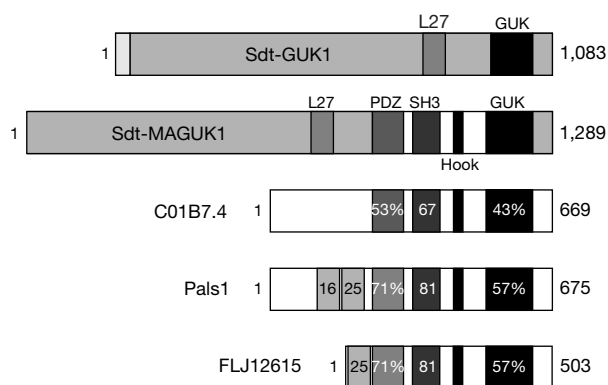


Figure 3 Proteins predicted from the *sdt* locus. Proteins predicted by cGUK1 (GenBank accession number AF414118) and cMAGUK1 (AF414117), *C. elegans* ORF C01B7.4 (AAA96115), mouse Pals1 (AF199008)⁸ and the human ORF FLJ12615 (BAB14172). Varying shades of grey indicate different protein domains. Amino-acid identities of 71, 81 and 57% in the PDZ, SH3 and GUK domains, respectively, suggest that Sdt MAGUK1 and the mammalian proteins are true orthologues.

cDNA cMAGUK1 (Fig. 2b) detects three developmentally regulated transcripts of about 5, 6 and 7 kb, respectively (Fig. 2c). Data from *in vitro* transcription/translation and overexpression of an upstream activating sequence (UAS)-cMAGUK1 transgene in wild-type embryos suggest that the first AUG codon, which complies to the Kozak consensus sequence, is used as the start site, resulting in a protein product (Sdt-MAGUK1) of relative molecular mass about 140,000 ($M_r \sim 140K$) (Fig. 2d, lanes 1 and 3). The protein contains a single PDZ (PSD-95, Discs Large, ZO-1) domain, an SH3 (Src homology region 3) domain and a GUK (guanylate kinase) domain (Fig. 3 and Supplementary Information), which, together with a Hook domain, classify Sdt-MAGUK1 as a member of the p55 subfamily of the MAGUK (membrane-associated guanylate kinase homologues) family of scaffolding proteins⁷. The MAGUK domain exhibits a high degree of similarity to that of a predicted open reading frame (ORF) from *Caenorhabditis elegans*, to mouse Pals1 (ref. 8) and to a human predicted ORF (Fig. 3). Sdt-MAGUK1 also shares the putative Lin-7-binding domain (L27) with the mammalian proteins.

Using cMAGUK1 as probe, the 4.5-kb cDNA cGUK1 was isolated, which starts about 25 kb downstream of the start site of cMAGUK1

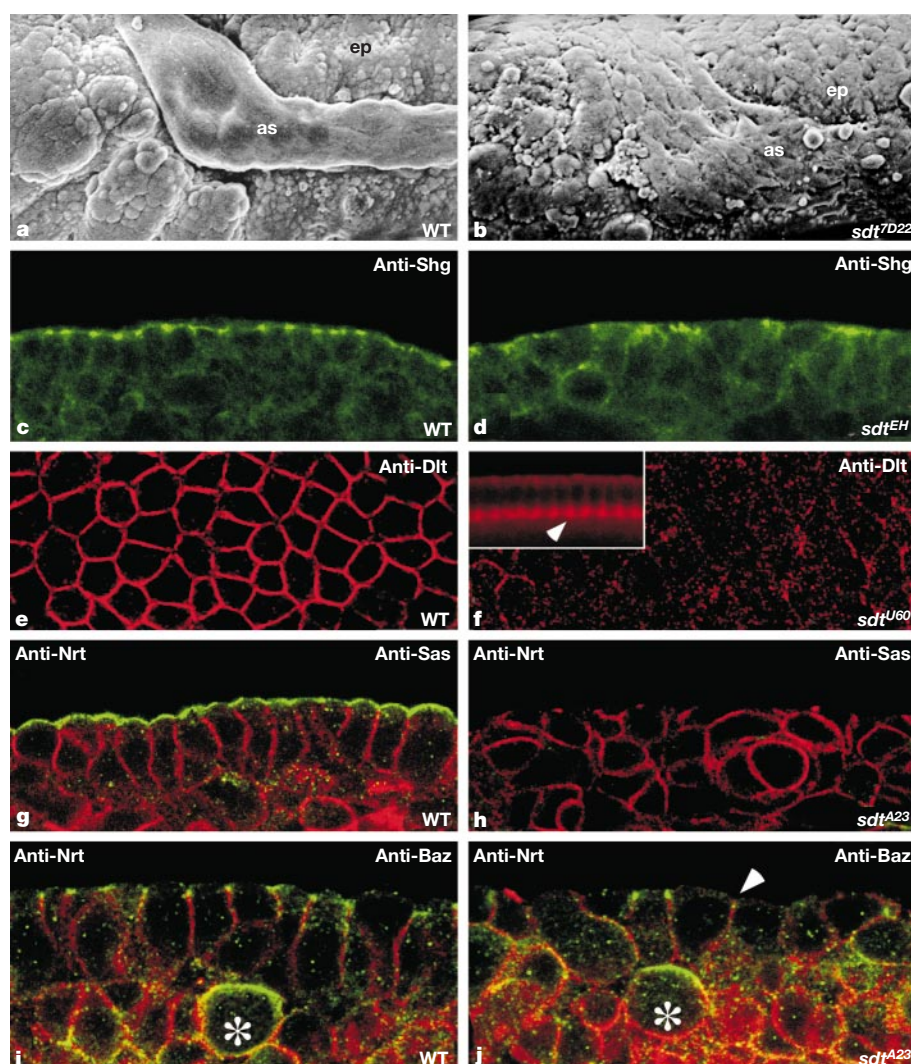


Figure 4 Phenotypic characteristics of *sdt* mutant embryos. **a, c, e, g, i**, Wild type (WT). **b, d, f, h, j**, *sdt* mutant embryos. **a, b**, Scanning electron micrographs of lateral views of stage 11 embryos. ep, epidermis; as, amnioserosa. **c, d**, Lateral optical sections of stage 11 embryos stained with anti-Shotgun/DE-cadherin, a constituent of the ZA. **e, f**, Surface views of stage 9 embryos stained with anti-Discs Lost, a component of the SAC. Inset (**f**), Dlt localization during cellularization. **g, h**, Lateral optical sections of stage

12 embryos stained with anti-Neurotactin (Nrt; red), and anti-Stranded at Second (Sas; green) basolateral and apical markers, respectively. **i, j**, Lateral optical section of stage 11 embryos, stained with anti-Neurotactin (Nrt; red) and anti-Bazooka (Baz; green). Asterisks indicate delaminated neuroblasts. In **a** and **b**, anterior is left, ventral down. In **c, d** and **g-j**, apical is up, basal down.

(Fig. 2a, b). A protein product (Sdt-GUK1) with M_r about 120K could be synthesized from cGUK1 *in vitro* (Fig. 2d, lane 2). It differs from Sdt-MAGUK1 by 78 additional amino-terminal amino acids and the lack of 284 internal amino acids, removing the PDZ, SH3 and Hook domains (Fig. 3 and Supplementary Information). Polymerase chain reaction with reverse transcription (RT-PCR) on embryonic poly(A)⁺ RNA was performed to verify the transcript represented by cGUK1. RT-PCR with primers from the cGUK1-specific 5' exon and the common 3' untranslated region (UTR), followed by a nested PCR with a primer pair flanking the MAGUK/GUK domain (Fig. 2f), yielded, besides a minor band of 1.3 kb that corresponds to a MAGUK transcript, one major product with the

expected size of 0.5 kb (Fig. 2e). Sequencing of this fragment revealed a slight difference compared with cGUK1 in the region between the Lin-7-binding domain and the GUK domain. From the sequence, a protein is predicted (named Sdt-GUK2) with a complete GUK domain and a deletion of 271 amino acids compared with Sdt-MAGUK1 (see Supplementary Information). These data suggest that *sdt* transcripts encode proteins with either a MAGUK or a GUK domain.

To link the transcripts characterized above to the *sdt* phenotype, injections of double-stranded RNA (dsRNA) into wild-type embryos (RNAi) were performed, using dsRNA from three different regions (Fig. 2f). In all three cases, weak to strong *sdt* phenotypes

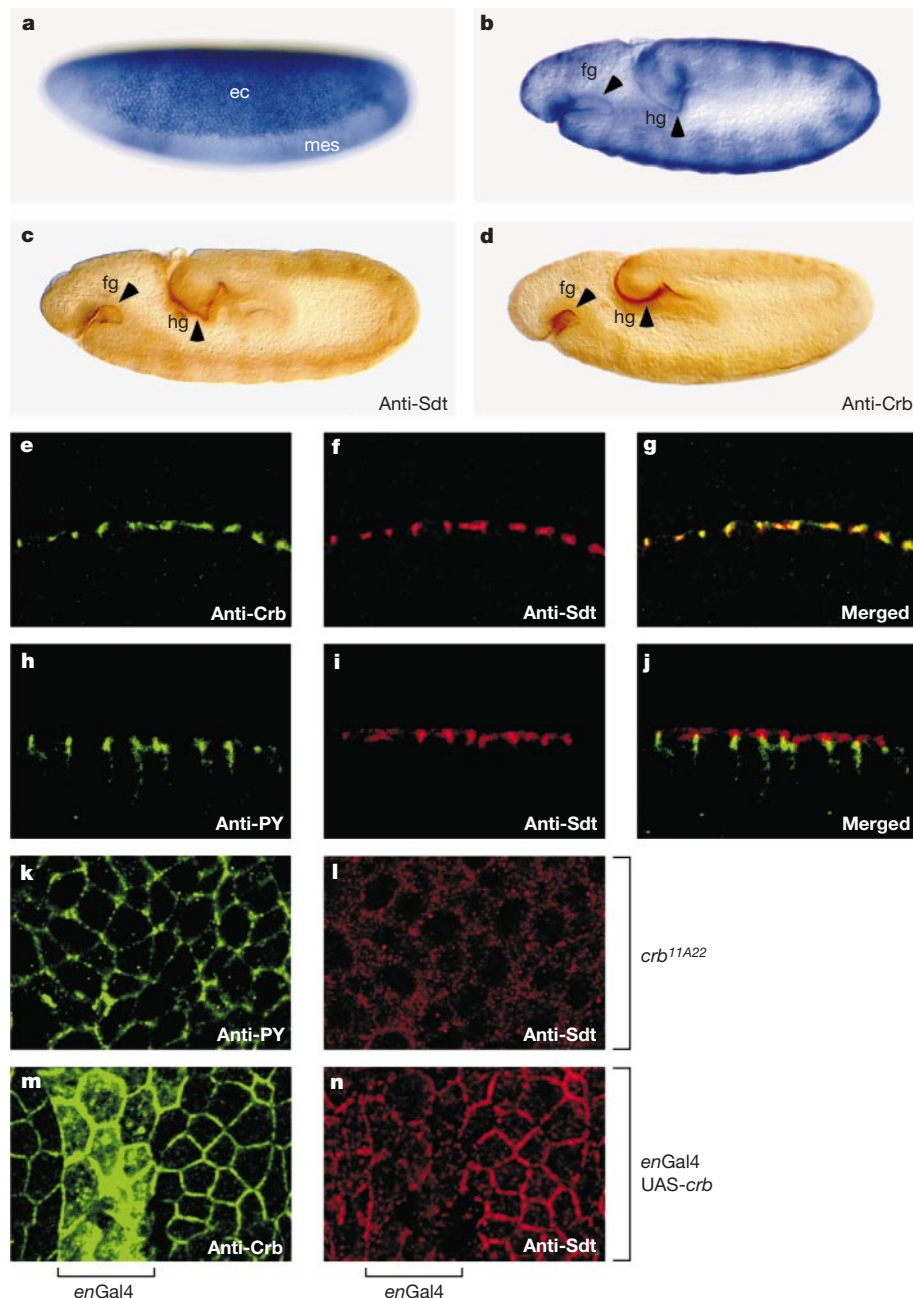


Figure 5 Relationship between *sdt* and *crb* expression. **a–d**, Expression of *sdt* RNA (**a, b**), Sdt protein (**c**) and Crb protein (**d**) in wild-type embryos of stages 6 (**a**) and 10 (**b–d**). ec, ectoderm; mes, mesoderm; fg, foregut; hg, hindgut. Arrowheads point to apical localization of RNA and proteins. **e–j**, Stage 13 (**e–g**) and stage 11 (**h–j**) wild-type embryos stained with anti-Crb (**e**; green), anti-Sdt (**f, i**; red) and anti-phosphotyrosine

(**h**; green), a marker for the ZA. Sdt is colocalized with Crb (**g**) apical to the ZA (**j**). **k–n**, Stage 12 *crb*^{11A22} embryos (**k, l**) and wild-type embryos overexpressing Crb in the posterior compartment of each segment (bar; **m, n**), stained with anti-phosphotyrosine (**k**; green), anti-Crb (**m**; green) and anti-Sdt (**l, n**; red) antibodies. In **a–d**, anterior is left, ventral down. In **e–j**, apical is up, basal down.

were observed, which ranged from holes in the cuticle to only grains of cuticle (Fig. 1d, e). To further assay that the cloned gene corresponded to *sdt*, we carried out rescue experiments. GAL4-mediated overexpression of UAS-cGUK1 did not rescue the *sdt* loss-of-function phenotype, whereas expression of UAS-cMAGUK1 gave considerable rescue (Fig. 1f), which was not significantly improved on overexpression of both transgenes. In summary, the data show that MAGUK1 represents a *sdt* gene product, whereas the function of the GUK proteins remains elusive.

The *sdt* mutant phenotype is characterized by the loss of epithelial integrity and cell shape (Fig. 4a, b). *sdt* mutant embryos fail to concentrate the scattered spot adherens junctions into a continuous ZA^{5,6,9} (Fig. 4c, d). As a consequence, cells lose contact and the epithelium becomes multilayered (Fig. 4a–d and g–j). As previously shown, the assembly of the ZA requires a functional SAC. Formation of the SAC depends on an intact cytoplasmic tail of Crumbs, which recruits the PDZ protein Discs Lost (Dlt) into the complex³. In *sdt* mutant embryos, the SAC components Crumbs (Crb)⁴ and Dlt (Fig. 4e, f) fail to localize apically from stage 8 onwards. Because Dlt is correctly associated with the ingrowing plasma membrane during cellularization in *sdt* embryos (Fig. 4f, inset), we suggest that *sdt* is, similarly to *crb*, essential for maintenance, but not for initiation of Dlt localization. The transmembrane protein Stranded at Second (Sas) is lost from the apical pole, whereas the basolateral marker Neurotactin, initially retained at the lateral membranes, is delocalized in later stages (Fig. 4g, h). Lack of *sdt* also affects localization of the PDZ protein Bazooka (Baz) in epithelial cells (Fig. 4i, j). Baz forms, together with the PDZ protein DmPar-6 and an atypical protein kinase C (DaPKC), another apical protein complex. This complex is essential for the polarity of epithelial cells and neuroblasts, the progenitors of the central nervous system, where it controls spindle orientation and localization of determinants^{10–13}. Lack of *sdt* function does not affect Baz localization in neuroblasts (Fig. 4j), showing that *sdt*, unlike *baz*^{10,11}, is not required for the apicobasal polarity of neuroblasts.

In the embryo, *sdt* transcripts can be detected from the cellular blastoderm onwards, where they are restricted to the anlage of the ectoderm (prospective ectoderm) and excluded from that of the mesoderm (Fig. 5a). Later on, *sdt* transcripts are present in epithelial derivatives of the ectoderm, that is, the epidermis, the foregut and hindgut, the tracheae, the salivary glands and the Malpighian tubules, where they are localized apically (Fig. 5b). At no time is *sdt* RNA detectable in neuroblasts or other cells of the central nervous system. Both the tissue distribution and the subcellular localization of *sdt* transcripts are similar to those of *crb* transcripts¹⁴.

We raised an antiserum against two synthetic peptides of the GUK domain, which thus should recognize both Sdt-MAGUK1 and Sdt-GUK1/2. It is specific for Sdt, because no staining was observed in several *sdt* alleles (data not shown). Apical staining was detected from gastrulation (stage 7) onwards in all epithelia derived from the ectoderm. Hence, Sdt shows the same temporal and spatial expression pattern as Crb (Fig. 5c, d). Sdt is colocalized with Crb in the SAC, apical to the ZA (Fig. 5e–j). In *crb* mutants, Sdt protein is lost from the plasma membrane (Fig. 5l), whereas some residual ZA components show a scattered distribution on all membranes (Fig. 5k). Sdt in *crb* mutants thus behaves similarly to Crb protein in *sdt* mutants⁴. Overexpression of Crb results in the depletion of Sdt from the apical surface (Fig. 5m, n).

These results and the previously shown genetic interaction between *crb* and *sdt*⁴ prompted us to look for a possible direct interaction between the two proteins using the yeast two-hybrid system. A fusion protein consisting of the MAGUK domain of Sdt and the transactivation domain of GAL4 was co-expressed in yeast cells with various forms of the Crb cytoplasmic domain, fused to the DNA-binding domain of GAL4. The full-length intracellular domain of Crb and those with mutations in a region close to the membrane (Crb-intra^{WT}, Crb-intra^{Y10A} and Crb-intra^{E16A}) showed

strong interaction with the MAGUK domain of Sdt. In contrast, lack of the four C-terminal amino acids ERLI (Crb-intra^{ΔERLI}) completely abolished the interaction (see Supplementary Information). This suggests that binding of the MAGUK domain of Sdt depends on the four C-terminal amino acids of Crb.

The data presented here suggest that *sdt* encodes several isoforms, which are constituents of the SAC. The presence of Sdt proteins containing either a MAGUK or a GUK domain has, to our knowledge, no precedent among other MAGUK proteins. Recent results have shown that the GUK domain of the rat MAGUK proteins SAP97 and PSD-95/SAP-90 (refs 15–17) and of hCASK and hDlg¹⁸ can form intra- as well as intermolecular interactions with the SH3 domain of the same or other MAGUK proteins, respectively. It has also been demonstrated that intramolecular interactions between the SH3 and the GUK domain of SAP97 modulate binding of its GUK domain to its partner GKAP.

Several issues are yet to be solved: how Crb, Dlt and Sdt participate in the organization of the SAC; in which temporal sequence they are recruited; and whether Dlt and Sdt compete for the Crb binding site. Sdt and Dlt behave differently on overexpression of Crb, in that Dlt is redistributed around the plasma membrane³, whereas Sdt is depleted from the apical membrane. The different Sdt isoforms now make it possible to isolate additional components of the SAC. The mouse homologue Pals1 has been isolated by virtue of its interaction with mouse Lin-7 (ref. 8), another PDZ protein. Homologues of Lin-7 and its *C. elegans* binding partners Lin-2 and Lin-10 have been identified in *Drosophila*¹⁹ (A.B. and E.K., unpublished data) and we are interested to see whether they form part of the SAC. The analysis of all the interactions necessary to establish a functional SAC, the control of its assembly and its role in the establishment of the ZA will allow further unravelling of the mechanisms involved in the control of apicobasal polarity of epithelial cells. □

Methods

Fly stocks

The following fly stocks were used: wild type (Oregon R), *crb*^{11A22}, *sdt*^{7D22}, *sdt*^{EH} (ref. 4), *sdt*^{U60}, *sdt*^{A23} (ref. 20), *sdt*^{P10} (this work), *EP(X)1143* (ref. 21), *Is(1)P(ry⁺)³³⁴* (ref. 22), *Gal4^{daG32}*, *UAS-cr^{30.12c}* (ref. 23), *enGal4* (ref. 24), *UAS-cMAGUK1* and *UAS-cGUK1* (this work).

Molecular analysis of *stardust*

The homozygous viable P-element insertion line *Is(1)P(ry⁺)³³⁴* was used as a starter strain to initiate a local hop mutagenesis with Δ2–3 as the source of transposase²⁵. A total of 7,250 single crosses were performed and 20 lethal P-element insertion lines could be isolated. By phenotypic and complementation analysis one P-element insertion turned out to be a new *sdt* allele, named *sdt*^{P10}. Mobilization of this P-element reverted the *sdt* mutant phenotype to wild type, demonstrating that the *sdt* mutation is caused by the P-element insertion.

Genomic DNA adjacent to the P-element insertion site in *sdt*^{P10} was isolated from a partial genomic phage library by screening with a P-element-specific probe. A chromosomal walk was initiated, isolating about 60 kb of flanking genomic DNA. Complementary DNAs from this region were recovered by screening the following cDNA libraries with various genomic fragments: NB 4–8-h embryonic library²⁶, Hovemann 0–16-h embryonic library²⁷, LD 0–22-h embryonic library (BDGP, provided by C. M. Schuster), and testis-specific library (provided by M. Schäfer). Two nearly full-length cDNAs were recovered: cMAGUK1 is 5,758 nucleotides long and has an ORF of 3,870 nucleotides; cGUK1 is 4,548 bp long and includes an ORF of 3,252 nucleotides.

RT-PCR, *in vitro* transcription/translation and RNAi

We performed RT-PCR using the Qiagen OneStep RT-PCR Kit with primers for *l* and *rev1*, followed by nested PCR with primers for *2* and *rev2* (Fig. 2f; for primer sequences see Supplementary Information). *In vitro* transcription/translation was carried out with the TNT Coupled Reticulocyte Lysate Systems (Promega). Double-stranded RNA for RNAi was produced according to the PCR template method and injected into the posteroventral region of preblastoderm embryos²⁸. The following primers were used for generation of dsRNA (Fig. 2f; for primer sequences see Supplementary Information): MAGUK-RNAi-5 and MAGUK-RNAi-3 (MAGUK-RNAi dsRNA inactivates proteins containing either a MAGUK or a GUK domain); SH3-RNAi-5 and SH3-RNAi-3 (SH3-RNAi dsRNA inactivates proteins containing a MAGUK domain); N-term-RNAi-5 and N-term-RNAi-3 (N-term-RNAi dsRNA inactivates proteins containing the cGUK1-specific 5' exon).

Germline transformation

Transgenic UAS-cMAGUK1 and UAS-cGUK1 flies were generated by introducing the corresponding cDNAs into *w*¹¹¹⁸ flies²⁹. Several transgenic lines were established for each construct.

In situ hybridization

A digoxigenin-labelled RNA antisense probe that recognizes a 3' part of cMAGUK1 encoding the PDZ, SH3 and GUK domains was generated using the DIG RNA Labeling Kit (Roche). *In situ* hybridization was performed following standard procedures.

Antibody production and immunohistochemistry

Two peptides from the C terminus of Sdt that are present in Sdt-MAGUK1 and Sdt-GUK1/2 (DKLRQKKLRNGEPFK and QWVPAQWVHNRRDES) were used to immunize rabbits (Eurogentec). For immunohistochemistry and western analysis anti-Sdt^{pep153} antibody was affinity purified and used at a dilution of 1:100 and 1:500, respectively. Enhancement of anti-Sdt^{pep153} antibody staining was accomplished with the Vectastain Elite ABC kit (Vector).

For immunohistochemistry, embryos were fixed by standard protocols³. The following antibodies were used for antibody staining: mouse anti-Crb Cq4 (ref. 4) (1:2), rabbit anti-Dlt² (1:1,000), mouse anti-phosphotyrosine PY20 (1:500, Transduction Laboratories), rabbit anti-Sas (1:500, E. Organ and D. Cavener, unpublished material), mouse anti-Nrt⁹ (1:10; Developmental Studies Hybridoma Bank), rat anti-Baz N-term¹¹ (1:200), rat anti-Shg³⁰ (DCad2, 1:20) and rabbit anti-Sdt^{pep153} (this work). Cy2- and Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch. HRP-conjugated secondary antibodies were from Dianova. Embryos were analysed with a Leica TCS NT confocal microscope and images were processed and mounted using Photoshop 5.5 (Adobe) and Canvas 6.0 (Deneba).

Scanning electron microscopy

Embryos were initially fixed in 25% glutaraldehyde in 0.05 M phosphate buffer and heptane (1:1). After extensive washing with 0.05 M phosphate buffer, embryos were further fixed on ice for 30 min with 2% osmium tetroxide in PBS, followed by several washes with PBS and dehydration with ethanol. Embryos were critical-point dried and covered with a gold-palladium layer 40–50 Å thick. Pictures were taken with a Hitachi S-520 scanning electron microscope.

Yeast two-hybrid interaction assays

A fragment from cMAGUK1 covering PDZ, SH3 and GUK domains was amplified by PCR using primers M5-Prot.5 and M5-Prot.3 (for primer sequences see Supplementary Information) and cloned in frame with the Gal4 transactivation domain into pACT2 (Clontech). The intracellular domain of Crb as well as mutant versions of it³ were amplified by PCR using primers crb5-intra and crb3-intra (for primer sequences see Supplementary Information) and cloned in frame with the Gal4 DNA-binding domain into pGBT9 (Clontech). Two-hybrid interaction assays were performed according to the manufacturer's instructions.

Received 19 June; accepted 24 September 2001.

- Müller, H. A. J. Genetic control of epithelial cell polarity: lessons from *Drosophila*. *Dev. Dyn.* **218**, 52–67 (2000).
- Bhat, M. A. *et al.* Discs Lost, a novel multi-PDZ domain protein, establishes and maintains epithelial polarity. *Cell* **96**, 833–845 (1999).
- Klebes, A. & Knust, E. A conserved motif in Crumbs is required for E-cadherin localisation and zonula adherens formation in *Drosophila*. *Curr. Biol.* **10**, 76–85 (2000).
- Tepass, U. & Knust, E. Crumbs and stardust act in a genetic pathway that controls the organization of epithelia in *Drosophila melanogaster*. *Dev. Biol.* **159**, 311–326 (1993).
- Grawe, F., Wodarz, A., Lee, B., Knust, E. & Skar, H. The *Drosophila* genes *crumbs* and *stardust* are involved in the biogenesis of adherens junctions. *Development* **122**, 951–959 (1996).
- Tepass, U. Crumbs, a component of the apical membrane, is required for zonula adherens formation in primary epithelia of *Drosophila*. *Dev. Biol.* **177**, 217–225 (1996).
- Dimitratos, S. D., Woods, D. F., Stathakis, D. G. & Bryant, P. J. Signaling pathways are focused at specialized regions of the plasma membrane by scaffolding proteins of the MAGUK family. *Bioessays* **21**, 912–921 (1999).
- Kamberov, E. *et al.* Molecular cloning and characterization of Pals, proteins associated with mLin-7. *J. Biol. Chem.* **275**, 11425–11431 (2000).
- Müller, H. A. & Wieschaus, E. *armadillo*, *bazooka*, and *stardust* are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in *Drosophila*. *J. Cell Biol.* **134**, 149–163 (1996).
- Schober, M., Schaefer, M. & Knoblich, J. A. Bazooka recruits Inscuteable to orient asymmetric cell divisions in *Drosophila* neuroblasts. *Nature* **402**, 548–551 (1999).
- Wodarz, A., Ramrath, A., Kuchinke, U. & Knust, E. Bazooka provides an apical cue for Inscuteable localization in *Drosophila* neuroblasts. *Nature* **402**, 544–547 (1999).
- Wodarz, A., Ramrath, A., Grimm, A. & Knust, E. *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J. Cell Biol.* **150**, 1361–1374 (2000).
- Petronczki, M. & Knoblich, J. A. DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in *Drosophila*. *Nature Cell Biol.* **3**, 43–49 (2001).
- Tepass, U., Theres, C. & Knust, E. *crumbs* encodes an EGF-like protein expressed on apical membranes of *Drosophila* epithelial cells and required for organization of epithelia. *Cell* **61**, 787–799 (1990).
- McGee, A. W. & Bredt, D. S. Identification of an intramolecular interaction between the SH3 and guanylate kinase domains of PSD-95. *J. Biol. Chem.* **274**, 17431–17436 (1999).
- Shin, H., Hsueh, Y.-L., Yang, F.-C., Kim, E. & Sheng, M. An intramolecular interaction between Src homology 3 domain and guanylate kinase-like domain required for channel clustering by post-synaptic density-95/SAP90. *J. Neurosci.* **20**, 3580–3587 (2000).

- Wu, H. *et al.* Intramolecular interactions regulate SAP97 binding to GKAP. *EMBO J.* **19**, 5740–5751 (2000).
- Nix, S. L., Chishti, A. H., Anderson, J. M. & Walther, Z. hCASK and hDlg associate in epithelia, and their Src homology 3 and guanylate kinase domains participate in both intramolecular and intermolecular interactions. *J. Biol. Chem.* **275**, 41192–41200 (2000).
- Dimitratos, S. D., Woods, D. F. & Bryant, P. J. Camguk, Lin-2, and CASK: novel membrane-associated guanylate kinase homologs that also contain CaM kinase domains. *Mech. Dev.* **63**, 127–130 (1997).
- Lammel, U. & Saumweber, H. X-linked loci of *Drosophila melanogaster* causing defects in the morphology of the embryonic salivary gland. *Dev. Genes Evol.* **10**, 525–535 (2000).
- Rorth, P. A modular misexpression screen in *Drosophila* detecting tissue-specific phenotypes. *Proc. Natl Acad. Sci. USA* **93**, 12418–12422 (1996).
- Rubin, G. M. & Spradling, A. C. Vectors for P-element-mediated gene transfer in *Drosophila*. *Nucleic Acids Res.* **11**, 6341–6351 (1983).
- Wodarz, A., Hinz, U., Engelbert, M. & Knust, E. Expression of Crumbs confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. *Cell* **82**, 67–76 (1995).
- Han, K. & Manley, J. L. Functional domains of the *Drosophila* Engrailed protein. *EMBO J.* **12**, 2723–2733 (1993).
- Tower, J., Karpen, G. H., Craig, N. & Spradling, A. C. Preferential transposition of *Drosophila* P-elements to nearby chromosomal sites. *Genetics* **133**, 347–359 (1993).
- Brown, N. H. & Kafatos, F. C. Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* **203**, 425–437 (1988).
- Hovemann, B. T., Dessen, E., Mechler, H. & Mack, E. *Drosophila* snRNP associated protein P11 which specifically binds to heat shock puff 93D reveals strong homology with hnRNP core protein A1. *Nucleic Acids Res.* **19**, 4909–4914 (1991).
- Kennerdell, J. R. & Carthew, R. W. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the Wiggless pathway. *Cell* **95**, 1017–1026 (1998).
- Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401–415 (1993).
- Oda, H., Uemura, T., Harada, Y., Iwai, Y. & Takeichi, M. A *Drosophila* homolog of cadherin associated with armadillo and essential for embryonic cell–cell adhesion. *Dev. Biol.* **165**, 716–726 (1994).

Supplementary Information accompanies the paper on Nature's website (<http://www.nature.com>).

Acknowledgements

We thank N. Brown, B. Hovemann, M. Schäfer and C. M. Schuster for cDNA libraries, M. A. Bhat, D. Cavener and T. Uemura for antibodies, A. Ramrath for help with the yeast two-hybrid assay, and J. A. Campos-Ortega, K. Johnson, A. Müller and A. Wodarz for discussions and critical reading of the manuscript. The work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

Correspondence and requests for materials should be addressed to E.K. (e-mail: knust@uni-duesseldorf.de).

Kinesin-mediated axonal transport of a membrane compartment containing β -secretase and presenilin-1 requires APP

Adeela Kamal*[†], Angels Almenar-Queralt*, James F. LeBlanc[‡], Elizabeth A. Roberts* & Lawrence S. B. Goldstein*

* Howard Hughes Medical Institute and Department of Cellular and Molecular Medicine, School of Medicine, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-0683, USA

[‡] CIPHERgen Biosystems Inc., 6611 Dunbarton Circle, Fremont, California 94555, USA

Proteolytic processing of amyloid precursor protein (APP) generates amyloid- β peptide and has been implicated in the pathogenesis of Alzheimer's disease¹. However, the normal function of APP, whether this function is related to the proteolytic processing of APP, and where this processing takes place in neurons *in vivo* remain unknown. We have previously shown that the axonal transport of APP in neurons is mediated by the direct binding

[†] Present address: Conforma Therapeutics Corporation, 9393 Towne Centre Drive, Suite 240, San Diego, California 92121, USA.

of APP to the kinesin light chain subunit of kinesin-I, a microtubule motor protein². Here we identify an axonal membrane compartment that contains APP, β -secretase and presenilin-1. The fast anterograde axonal transport of this compartment is mediated by APP and kinesin-I. Proteolytic processing of APP can occur in the compartment *in vitro* and *in vivo* in axons. This proteolysis generates amyloid- β and a carboxy-terminal fragment of APP, and liberates kinesin-I from the membrane. These results suggest that APP functions as a kinesin-I membrane receptor, mediating the axonal transport of β -secretase and presenilin-1, and that processing of APP to amyloid- β by secretases can occur in an axonal membrane compartment transported by kinesin-I.

The principal processing enzymes that generate amyloid- β (A β) from APP are thought to be β -secretase (Bace)³ and γ -secretase, proposed to be encoded by the presenilin genes^{4,5}. Presenilins are also involved in Notch proteolysis^{6,7}. Whether presenilins are proteases that cleave APP and Notch, or whether presenilins are a crucial cofactor, is controversial, as is the cellular site of APP processing. Presenilin-1 (Psl, also known as Psen1) is found in

endoplasmic reticulum (ER) and pre-Golgi compartments^{8,9}, whereas A β generation is reported to occur in the endosomal system¹⁰, the *trans*-Golgi network^{11,12} and the ER^{13,14}, although the latter site is controversial^{15,16}. Most of these studies used cultured cells, and thus it is unknown how these findings relate to neurons *in vivo*, whether APP, Bace and presenilin are ever in the same membrane compartment, and, if so, where and why. The normal cellular functions of APP are also unclear. Mice deficient in APP, although viable, are abnormal and have reduced weight, decreased neuromuscular performance, reactive gliosis in the cortex and hippocampus, reduced synaptic plasticity, loss of synaptic immunoreactivity for the vesicle proteins synapsin and synaptophysin, and defects in the corpus callosum^{17–20}. However, the cellular basis for the defects caused by loss of APP is unknown. Our previous finding², that axonal transport of APP seems to be mediated by binding of APP to kinesin light chain (Klc), suggested that one function of APP may be as a kinesin-I membrane receptor for the axonal transport of an unknown membrane compartment.

To investigate the role of APP in axonal transport, we compared the steady-state levels of kinesin-I and putative kinesin-I cargoes in sciatic nerve axons, the dorsal root ganglion (DRG), and corpus callosum from APP-null mutant and wild-type mice (Fig. 1a, Table 1). Sensory and motor axons are a major component of sciatic nerves; thus, abundance of a neuronal protein in this tissue gives an estimate of its transport into axons². We observed a significant reduction of the neuronally enriched Klc (Klc1), ubiquitous Klc (Klc2), and the ubiquitous kinesin heavy chain (Kif5b), subunits of kinesin-I in the sciatic nerves of APP-null mice (Fig. 1a). There was a corresponding decrease of Gap43 and synapsin-I, two previously proposed kinesin-I cargoes^{2,21}. We also examined several proteins whose transport was not previously tested for kinesin-I dependence and found a reduced amount of Bace, Psl and the neurotrophin receptor TrkA (tyrosine kinase receptor A, also known as Ntrk1), in sciatic nerves from Klc1-knockout mice (data not shown) and APP-null mice (Fig. 1a). The amount of synaptotagmin and synaptophysin, cargoes of a different kinesin-like motor protein^{22,23}, remained unchanged in sciatic nerves of the APP-null mutant, as did the retrograde motor protein dynein and a different anterograde motor protein, kinesin-II (Fig 1b, Kif3a). We also examined the DRG, which is primarily composed of cell bodies that supply sciatic nerve sensory axons with transported proteins. In the APP-null mutant, we observed an increase in kinesin-I (Kif5b and Klc), Bace, Psl, Gap43, synapsin-I and TrkA (Fig 1a, Table 1). Synaptotagmin, synaptophysin and dynein remained unchanged. To extend our findings to the central nervous system (CNS), we examined corpus callosum, which is primarily composed of CNS axons. We observed significant decrease in kinesin-I, Bace, Psl, Gap43, synapsin-I and TrkA in corpus callosum from APP-null

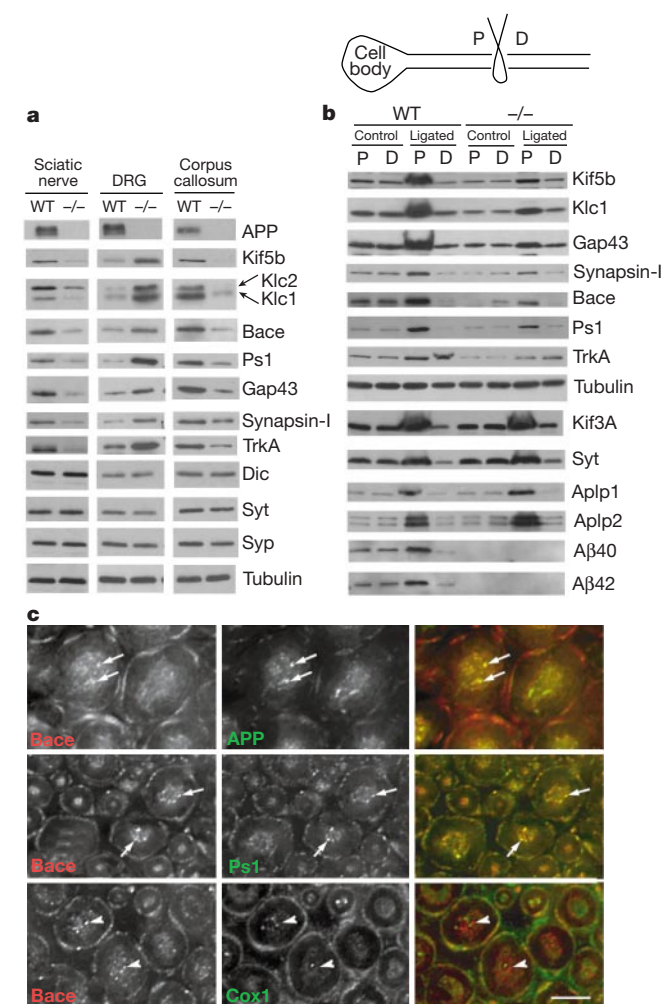


Figure 1 Axonal transport of Bace, Psl and other kinesin-I cargoes is decreased in APP-null mutant mice. **a**, Equal protein extracts from sciatic nerve, DRG and corpus callosum of age-matched wild-type (WT) and APP-mutant (–/–) mice. **b**, Ligated sciatic nerves from wild-type and APP-mutant mice; proximal (P) and distal (D) halves of the unligated (control) and ligated nerves are shown. **c**, Deconvolution images of cross-sections of mouse sciatic nerves double-stained with antibodies to Bace and APP, Bace and Psl, or Bace and Cox1; merged images are shown on the right. Arrows show examples of colocalization of proteins; arrowheads show absence of colocalization. Scale bar, 5 μ m.

Table 1 Protein levels in tissues of APP-null mice				
	SN	DRG	CC	Prox. ligated
APP	0.00	0.00	0.00	0.00
Kif5b	0.15	3.63	0.15	0.23
Klc1	0.23	3.11	0.20	0.25
Klc2	0.31	3.15	0.35	0.31
Bace	0.23	2.87	0.30	0.29
Psl	0.18	3.32	0.26	0.27
Gap43	0.21	3.15	0.27	0.26
Synapsin-I	0.22	3.32	0.29	0.24
TrkA	0.28	2.80	0.28	0.29
Dic	0.96	1.15	0.91	0.98
Syt	0.92	1.01	0.87	1.19
Syp	0.94	0.91	0.90	0.97
Tubulin	1.00	1.00	1.00	1.00

Proteins were measured in sciatic nerve (SN), DRG, corpus callosum (CC) and the proximal side of ligated nerves (Prox. ligated). Quantitative western blots were done as described in the Methods. The relative normalized amount of each protein in wild type was set to 1.0 and the amount of each protein present in the APP-null mutant was determined with tubulin as an internal normalization and loading control. Syt, synaptotagmin; Syp, synaptophysin. Dic, dynein intermediate chain. Also see Fig. 1a, b.

mice, whereas dynein, synaptotagmin and synaptophysin remained unchanged (Fig. 1a, Table 1). Proteins that are reduced in sciatic nerve and corpus callosum are not generally destabilized by absence of APP because their abundance in total brain extracts of APP-null mice is normal (data not shown). Thus, reduced presence of these proteins in the sciatic nerve and corpus callosum, and increased presence in the DRG, may reflect reduced axonal transport in the APP-null mutant.

To directly assess axonal transport of potential APP-dependent cargoes, mouse sciatic nerves were ligated for varying times and nerve portions proximal or distal to the ligation were analysed by western blots. Proteins moving in anterograde axonal transport pathways generally accumulate on the proximal side, whereas stationary, slow-moving or nonaxonal proteins remain unchanged. As previously reported², we observed accumulation of Klc1 and Kif5b on the proximal side of nerve ligations of wild-type mice (Fig. 1b, Table 1). APP-null mice had significantly reduced accumulation of Klc1 and Kif5b on the proximal side of a ligation. Bace, Ps1, Gap43 and synapsin-I also accumulated substantially on the proximal side of a ligation in the wild type but were reduced in APP-null mice (Fig. 1b). TrkA increased in both the proximal and distal halves in the wild type and was decreased in both halves in the APP-null mutant. Control proteins, such as the slow transport marker tubulin, exhibited equal abundance in proximal and distal halves after ligation, whereas synaptotagmin, kinesin-II (Kif3a), the APP relatives Aplp1 and Aplp2, dynein and synaptophysin accumulated to comparable levels on the proximal side of a ligation in wild-type and APP-null mice (Fig. 1b and data not shown).

The decreased transport of kinesin-I and potential cargoes in APP-null sciatic nerves suggested that APP would be abundant relative to kinesin-I. Quantitative western blots with protein standards expressed in bacteria revealed that APP and kinesin-I are both about 1% of total sciatic nerve protein, which is higher than

previous measurements of APP (0.05%)²⁴ and kinesin-I (0.3%)²⁵ in whole brain. However, sciatic nerve is highly enriched in axons and is depleted of many cellular elements such as nuclei, translation machinery, and Golgi apparatus. Staining experiments confirm that most of the APP and kinesin-I in sciatic nerve is axonal and moving in fast anterograde axonal transport.

Immunofluorescence staining of ligation experiments in wild-type mice with APP, Ps1 and Bace antibodies revealed accumulation and significant colocalization of Bace and APP or Bace and Ps1 primarily on the proximal side of a ligation (Supplementary Information Fig. 1). Immunofluorescence staining for APP, Bace, Ps1 and kinesin-I in cross-sections of mouse sciatic nerves revealed that they were primarily localized in axons, with some staining detectable in Schwann cells (Supplementary Information Fig. 2). Deconvolution analysis revealed that the staining pattern for APP, Bace and Ps1 in axons appeared vesicular, and that Bace staining overlapped with both APP and Ps1, but not with the mitochondrial marker Cox1 (Fig. 1c).

To probe for an axonal membrane compartment containing APP, Bace and Ps1, we fractionated mouse sciatic nerves. A vesicle-enriched fraction (P3) contained APP, Bace and Ps1, was de-enriched in tubulin, neurofilament-H and mitochondrial markers, and lacked markers for Golgi or ER (Supplementary Information Fig. 3a). Electron microscopy of the P3 fraction revealed large quantities of membranous structures ranging in size from 50 to 200 nm that appeared vesicular in nature (data not shown). Although they are heterogeneous in morphology, for simplicity we refer to these membranous structures as vesicles. The P3 membranes containing APP, Bace and Ps1 floated to the 10–15% I2 interface of iodixanol step gradients (Fig. 2a). Immuno-isolation from the I2 interface with an APP carboxy-terminal, Klc or TrkA (an axonal marker) antibodies brought down a fraction containing kinesin-I, APP, Bace and Ps1 (Fig. 2c). The non-kinesin-I cargoes,

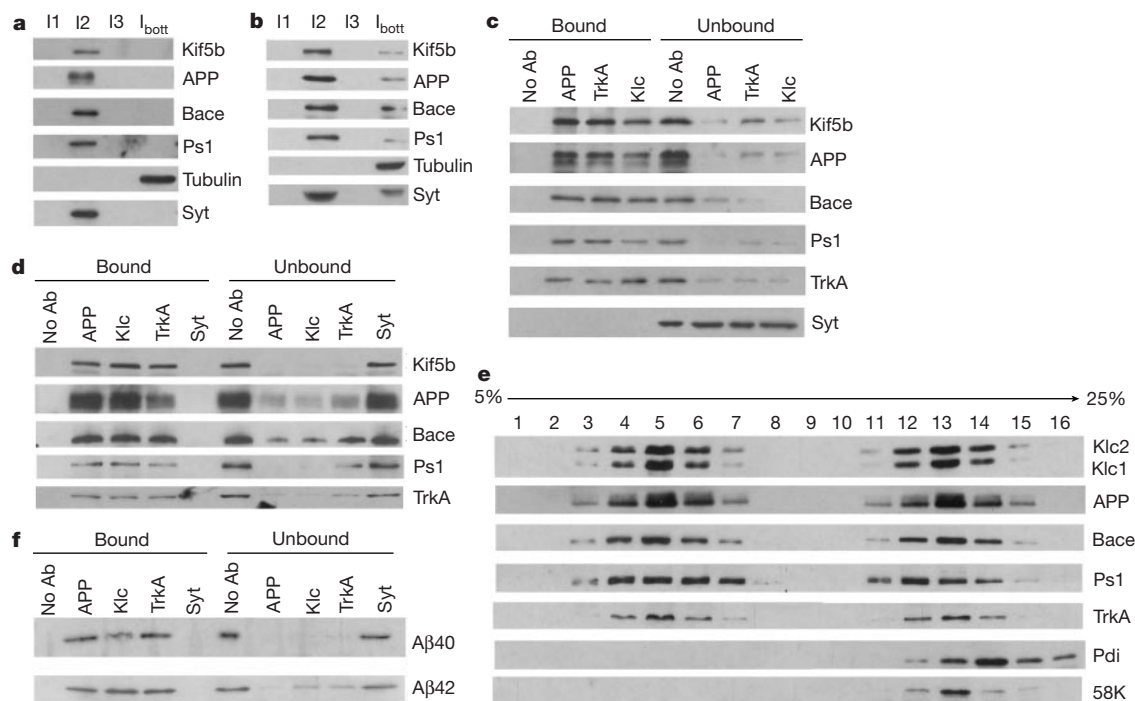


Figure 2 APP, Bace, Ps1 and kinesin-I are present in a specific subset of vesicles in mouse sciatic nerves and corpus callosum. **a, b**, Vesicles from sciatic nerves (**a**) or corpus callosum (**b**) were floated on an iodixanol step gradient and the interfaces 5–10% (I1), 10–15% (I2), 15–23% (I3) and the bottom of the gradient (I_{bottom}) were collected. **c, d**, Sciatic nerve (**c**) or corpus callosum (**d**) vesicles were immuno-isolated from the I2 interface of the iodixanol step gradient using either no antibody (No Ab) or antibodies to the

APP C terminus, Klc, TrkA and synaptotagmin (Syt). Equal amounts of bound (vesicle IP) and unbound vesicles were analysed. **e**, Two pools of kinesin-I, APP, Bace, and Ps1 are present in mouse cortex in a 5–25% linear iodixanol gradient. Equal volumes of the 16 collected fractions were analysed. **f**, Sciatic nerve vesicles were immuno-isolated from the I2 interface of the iodixanol step gradient and analysed by western blots with Aβ-specific antibodies.

synaptophysin (Supplementary Information Fig. 3b) and synaptotagmin, were absent from such immuno-isolations. An antibody recognizing synaptotagmin brought down synaptotagmin and synaptophysin as previously reported²², but not kinesin-I, Bace, Ps1, Gap43, synapsin-I or TrkA (Supplementary Information Fig. 3b). None of the antibodies brought down the ER marker, Bip/Grp78 (data not shown). Control immuno-isolation with no antibody did not bring down any marker protein. In addition, Aplp1 and Aplp2 were present in kinesin-I, but not in APP or synaptotagmin immuno-isolations (Supplementary Information Fig. 3b). When immuno-isolations from P3 were done in the presence of detergent, APP, kinesin-I, Aplp1, Aplp2 and Ps1 co-immunoprecipitated^{2,26}, whereas the association of Bace, Gap43, synapsin-I and TrkA with APP or kinesin-I was disrupted (Supplementary Information Fig. 3c).

To test for a membrane compartment containing APP, Bace and Ps1 in the CNS, we analysed mouse brain cortex. Like sciatic nerve, kinesin-I (Kif5b), APP, Bace and Ps1 co-enriched at the 10–15% I2 interface of an iodixanol step gradient of P3 cortical membranes (Fig. 2b). We extended this analysis by fractionating cortical membranes on a continuous 5–25% iodixanol gradient (Fig. 2e). We observed two pools of kinesin-I, APP, Bace and Ps1. One pool was near the bottom of the gradient and was characterized by the presence of ER (protein disulphide isomerase, PDI) and Golgi markers (relative molecular mass 58,000, M_r 58K protein). There was also a second, lighter pool that lacked these markers and had an estimated density similar to the 10–15% I2 component observed in

step gradients of sciatic nerve and cortical membranes. To test directly for a membrane compartment containing APP, Bace, Ps1 and kinesin-I in the CNS, we used immuno-isolation from the I2 interface of the step gradients (Fig. 2d). Antibodies to APP, Klc or TrkA co-immuno-isolated kinesin-I (Kif5b), TrkA, APP, Bace and Ps1, but not synaptotagmin. No antibody controls or a synaptotagmin antibody precipitated none of these proteins. The synaptotagmin antibody did bring down synaptotagmin and synaptophysin (data not shown), as in the sciatic nerve immuno-isolations.

Because our data suggest that the machinery for generation of A β from APP is present in a common axonal membrane compartment, we tested for A β in our preparations. We verified the specificity of the three A β antibodies we used by demonstrating that they could recognize and immunoprecipitate their respective synthetic peptides and by determining the mass of material immunoprecipitated by these antibodies by surface-enhanced laser desorption/ionization–time of flight (SELDI–TOF) mass spectrometry (Supplementary Information Fig. 4). In sciatic ligation experiments, we observed that in wild-type, but not APP-null mice, both A β 40 and A β 42 immunoreactive bands accumulated on the proximal side compared with the unligated control nerve (Fig. 1b). We also observed A β 40 and A β 42 in the sciatic nerve axonal vesicle fractions immuno-isolated from step-gradients with antibodies to APP, Klc and TrkA, but not synaptotagmin or no antibody controls (Fig. 2f).

To test whether A β 40 and A β 42 can be generated in axons, as opposed to being generated in the neuronal cell body and then transported into axons, we developed a double-ligation method for sciatic nerves. Wild-type mouse sciatic nerves were simultaneously ligated at the proximal and distal ends such that entry and exit of proteins into the region between the double ligation was blocked (Fig. 3a). A time-course experiment demonstrated a gradual increase in A β 40 and A β 42 between the 1-h, 2-h, 4-h and 6-h points accompanied by a corresponding decrease in full-length APP compared with unligated controls (Fig. 3a). Bace, Kif5b, Gap43, tubulin and synaptotagmin remained unchanged in the double-ligated nerves (Fig. 3a and data not shown), suggesting that there was not widespread proteolysis.

To determine whether A β 40 and A β 42 could be generated *in vitro* in the isolated fractions of the sciatic nerve vesicles, we warmed axonal vesicles to 37 °C for varying times (Fig. 3b). We observed a time-dependent generation of A β 40 and A β 42 in vesicles that were incubated at 37 °C compared with control vesicles frozen at –80 °C. There was also a time-dependent decrease of APP but not of Bace, Kif5b or synaptotagmin in vesicles incubated at 37 °C.

To investigate whether processing of APP had a role in the normal transport process, we examined whether cleavage of APP by γ -secretase would liberate the cytoplasmic C-terminal kinesin-I-binding region of APP and kinesin-I from membranes. We examined membrane-associated and soluble proteins from nerve extracts centrifuged after double ligation for 6 h. We observed significantly increased soluble kinesin-I (Kif5b, Klc1 and Klc2) after double ligation, whereas we found increased A β 40 and A β 42 in the membrane pellet fractions (Fig. 3c). In addition, a fragment of M_r about 8K was released into the supernatant. This fragment was recognized by an antibody specific for the APP C terminus, but not by the 4G8 antibody, which detects an epitope in the A β region of APP. Bace and synaptotagmin remained unchanged in the double-ligated nerve extracts and were found in membrane pellet fractions. We also tested for similar behaviour after APP proteolysis *in vitro*: kinesin-I (Kif5b, Klc1 and Klc2) and the APP C terminus were released into the supernatant whereas A β was found only in pellet fractions after incubation of vesicles *in vitro* at 37 °C (Fig. 3d). Bace and synaptotagmin remained unchanged and were found in membrane pellet fractions.

Our data confirm a role for APP as a kinesin-I membrane receptor needed for Bace and Ps1 transport in an axonal membrane compartment that may also contain Gap43, synapsin-I, and the

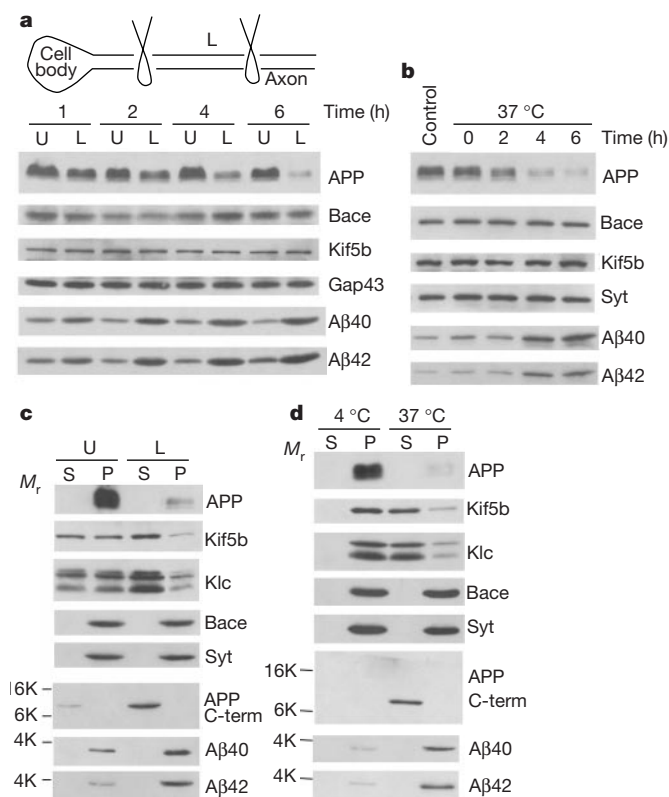


Figure 3 Time-dependent generation of A β and release of kinesin-I and the C terminus of APP. **a**, Double ligation of wild-type mouse sciatic nerves from 0 to 6 h leads to increased A β and decreased full-length APP. **b**, Warming sciatic nerve vesicles to 37 °C for 0–6 h compared with –80 °C (control) resulted in increased amounts of A β and corresponding decrease in full-length APP. **c**, **d**, Double ligation of mouse sciatic nerves for 6 h (**c**) and *in vitro* incubation of vesicles at 37 °C for 4 h (**d**) lead to release of kinesin-I and C terminus (C-term) of APP into supernatant (S), whereas A β and other membrane proteins remain in the pellet (P). Pellet and supernatant fractions were separated by centrifugation at 100,000g. Relative molecular mass markers are shown on left.

neurotrophin receptor Trk. The viability and incompletely penetrant phenotype of APP-null mice may be due to redundant receptor functions provided by Aplp1 or Aplp2 (Supplementary Information Fig. 3b, c), or by other unrelated kinesin-I receptor proteins^{27,28} mediating partially overlapping pathways. Our findings also suggest that an axonal membrane compartment may be an important *in vivo* site for proteolytic processing of APP to generate A β . Others have also found a pool of Ps1 not localized to the ER⁷ that does not cofractionate with ER markers in mouse cortical neurons and cultured cells²⁹. Together these data support the possibility that a 'non-ER' pool of presenilin might be located in a membrane-bound transport compartment containing other presynaptic proteins destined for the neuronal terminus.

Whether APP processing has any role in the normal transport process is not yet clear. However, liberation of the cytoplasmic C-terminal kinesin-I-binding region of APP and kinesin-I by γ -secretase cleavage might lead to either stalling or retrograde transport of vesicles back to the cell body. Such a process could also terminate kinesin-I transport at the axonal terminus or regulate vesicle transport after axonal damage. We suggest that cleavage of APP in response to axonal damage or blockage as shown here, or perhaps APP cleavage occurring at a slow spontaneous rate, might also cause A β deposition in axons, owing to the release of kinesin-I from moving vesicles in which A β is generated. If the degree of vesicle stalling and A β deposition is substantial, blockage of other axonal transport pathways critical for neurotrophic signalling might ensue, perhaps leading to neuronal death. Finally, it was recently reported³⁰ that the cytoplasmic APP C terminus might have a role in transcriptional induction, although what conditions might induce formation of the C terminus were not known. Our data raise the possibility that axonal damage might induce APP proteolysis to release the C terminus, which might then transmit an injury signal to the cell body. □

Methods

Antibodies and western blots

For western blots, the Klc (63–90 and Klc1), Kif5b, APP (22C11), APP (4G8), tubulin, dynein intermediate chain (Dic), Kif3a, synaptotagmin, synaptophysin and Gap43 antibodies were used as previously described². Additional antibodies used for western blots: APP C-terminal (Chemicon), synapsin-I (Stressgen), Cox1 (Molecular Probes), A β 40 (Chemicon) or A β 42 (Chemicon), TrkA (Research Diagnostics; this antibody may cross-react with TrkB and TrkC), neurofilament-H (Smi-31, Sternberger), Bace goat polyclonal (Bace1, Chemicon), Bace1 rabbit polyclonal (Oncogene), Bip (Grp78) (Stressgen) and Pdi (Affinity Bioreagents). The N-terminal presenilin-1 (NT) antibody, and the Aplp1 (Ct11) and Aplp2 (D2II) antibodies were provided by S. Sisodia. Presenilin-1 polyclonal antibody (Chemicon) gave the same results as the NT antibody from S. Sisodia; all blots shown are for NT. For immunofluorescence staining APP C-terminal (Chemicon), Bace goat polyclonal (Chemicon), presenilin-1 (NT), and Cox1 (Molecular Probes) antibodies were used. In general, samples were analysed by Tris–glycine SDS–PAGE and western blots; for the A β blots, Tris–tricine gels were used.

Protein in sciatic nerves, DRGs, corpus callosum

Sciatic nerves, DRGs or corpus callosum from three pairs of age-matched nonlittermate wild-type and APP-null mutant mice¹⁷ were dissected, quick frozen in liquid nitrogen, powdered with a pulverizer, resuspended in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH 6.8), centrifuged at 3,000g (to remove any particulate material) and the supernatant collected. Equal amounts of protein were analysed by SDS–PAGE and quantitative western blots, as previously described², with the indicated antibodies. Multiple exposures and dilutions were done to ensure that the antibodies were in the linear range; one representative experiment is shown, and the standard error was less than 5% from three independent experiments using three pairs of wild-type and APP-null mutant mice.

Sciatic nerve ligations

Sciatic nerve ligation experiments were done as previously described² except the proximal and distal halves of the nerves were processed as described above by quick freezing in nitrogen. For double-ligation experiments, sciatic nerves of wild-type mice were ligated at the most accessible proximal and distal ends simultaneously; the other nerve was left unligated as a control. One representative experiment is shown; similar results were obtained from four independent experiments using four pairs of wild-type and APP-null mice.

Immunofluorescence microscopy of sciatic nerve

Mouse sciatic nerves were dissected, embedded and frozen in methanol cooled with liquid

carbon dioxide; serial 10- μ m cryostat cross-sections were cut, fixed in 3.7% formaldehyde for 30 min at room temperature, washed, and incubated in blocking solution (3% BSA, 0.2% Triton-X100 in PBS) for 30 min. Sections were then incubated overnight at 4 °C with primary antibodies, washed, incubated with Texas Red or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies for 3 h at room temperature, and washed. The sections were mounted and observed with a 100 $\times$ oil-immersion objective with a Delta Vision Deconvolving microscope.

Fractionation of sciatic nerves and cortex

Vesicle-enriched (P3) fractions from mouse sciatic nerves or cortex were prepared by sequential centrifugation at 3000g (P1), 9,000g (P2) and 100,000g (P3) in 20 mM HEPES buffer at pH 7.3, 40 mM potassium chloride, 5 mM EGTA and 5 mM magnesium chloride with protease inhibitors. For flotation on iodixanol (Optiprep, Nycomed) step gradients, the P3 pellet was adjusted to 23% iodixanol, and overlaid with 1 ml each of 15%, 10% and 5% iodixanol in 10 mM HEPES at pH 7.3, 1 mM EDTA and 0.25 M sucrose with protease inhibitors. The gradients were centrifuged in a Sorvall TH-660 swinging bucket rotor at 38,000r.p.m. (150,000g) for 90 min at 4 °C. The interfaces at 5–10% (I1), 10–15% (I2) and 15–23% (I3), and the bottom of the gradient (I_{bot}), were collected. Equal amounts of protein were analysed by SDS–PAGE and western blots. For continuous gradients the P3 pellet from cortex was adjusted to 50% iodixanol, bottom loaded on a 5–25% iodixanol gradient, and then centrifuged as above. Sixteen fractions were collected, and equal volumes were analysed by SDS–PAGE and western blots.

Immuno-isolation of vesicles

Vesicles (P3) and the 10–15% I2 interface from step gradient I2 were prepared as described above from sciatic nerves or cortex, incubated for 1 h at 4 °C with indicated antibodies, and then protein A Sepharose beads added for 1 h at 4 °C. The beads were then centrifuged for 30 s at 3,000g and supernatant was saved as unbound vesicles. The beads were washed and resuspended in SDS sample buffer to release bound vesicles.

Generation of A β in sciatic nerve vesicles

Generation of A β was assayed *in vitro* by minor modification of a previously published procedure²⁶. Briefly, sciatic nerve vesicles (P3) were prepared as described above, and 1 M sodium citrate (pH 5.6) added to a final concentration of 50 mM. Fifty microlitres of vesicles were mixed with 50 μ l of 2 $\times$ stop solution (2% NP-40, 2 mM EDTA, 1 M guanidine hydrogen chloride and protease inhibitors), and incubated at –80 °C or 4 °C as the control reactions. For the experimental conditions, 50 μ l of vesicles were incubated at 37 °C for the indicated time, and then 50 μ l of 2 $\times$ stop solution were added.

Received 26 August; accepted 4 October 2001.

- Selkoe, D. J. Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* **399**, A23–A31 (1999).
- Kamal, A., Stokin, G. B., Yang, Z., Xia, C. & Goldstein, L. S. Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. *Neuron* **28**, 449–459 (2000).
- Vassar et al. β -secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735–741 (1999).
- De Strooper, B. et al. Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391**, 387–390 (1998).
- Selkoe, D. J. & Wolfe, M. S. In search of γ -secretase: presenilin at the cutting edge. *Proc. Natl Acad. Sci. USA* **97**, 5690–5692 (2000).
- De Strooper, B. et al. A presenilin-1-dependent γ -secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518–522 (1999).
- Ray, W. J. et al. Cell surface presenilin-1 participates in the γ -secretase-like proteolysis of Notch. *J. Biol. Chem.* **274**, 36801–36807 (1999).
- Annaert, W. G. et al. Presenilin 1 controls γ -secretase processing of amyloid precursor protein in pre-Golgi compartments of hippocampal neurons. *J. Cell Biol.* **147**, 277–294 (1999).
- Walter, J. et al. The Alzheimer's disease-associated presenilins are differentially phosphorylated proteins located predominantly within the endoplasmic reticulum. *Mol. Med.* **2**, 673–691 (1996).
- Koo, E. H. & Squazzo, S. L. Evidence that production and release of amyloid β -protein involves the endocytic pathway. *J. Biol. Chem.* **269**, 17386–17389 (1994).
- Thinakaran, G., Teplow, D. B., Siman, R., Greenberg, B. & Sisodia, S. S. Metabolism of the "Swedish" amyloid precursor protein variant in neuro2a (N2a) cells. Evidence that cleavage at the " β -secretase" site occurs in the Golgi apparatus. *J. Biol. Chem.* **271**, 9390–9397 (1996).
- Xu, H. et al. Generation of Alzheimer β -amyloid protein in the *trans*-Golgi network in the apparent absence of vesicle formation. *Proc. Natl Acad. Sci. USA* **94**, 3748–3752 (1997).
- Chyung, A. S. C., Greenberg, B. D., Cook, D. G., Doms, R. W. & Lee, V. M. Novel β -secretase cleavage of β -amyloid precursor protein in the endoplasmic reticulum/intermediate compartment of NT2N cells. *J. Cell Biol.* **138**, 671–680 (1997).
- Skovronsky, D. M., Doms, R. W. & Lee, V. M. Detection of a novel intraneuronal pool of insoluble amyloid β protein that accumulates with time in culture. *J. Cell Biol.* **141**, 1031–1039 (1998).
- Maltese, W. A. et al. Retention of the Alzheimer's amyloid precursor fragment C99 in the endoplasmic reticulum prevents formation of amyloid β -peptide. *J. Biol. Chem.* **276**, 20267–20279 (2001).
- Cupers, P. et al. The discrepancy between presenilin subcellular localization and γ -secretase processing of amyloid precursor protein. *J. Cell Biol.* **154**, 731–740 (2001).
- Zheng, H. et al. β -amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. *Cell* **81**, 525–531 (1995).
- Dawson, G. R. et al. Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the β -amyloid precursor protein. *Neuroscience* **90**, 1–13 (1999).
- Seabrook, G. R. et al. Mechanisms contributing to the deficits in hippocampal synaptic plasticity in mice lacking amyloid precursor protein. *Neuropharmacology* **38**, 349–359 (1999).

20. Magara, F. *et al.* Genetic background changes the pattern of forebrain commissure defects in transgenic mice underexpressing the β -amyloid-precursor protein. *Proc. Natl Acad. Sci. USA* **96**, 4656–4661 (1999).
21. Ferreira, A., Niclas, J., Vale, R. D., Banker, G. & Kosik, K. S. Suppression of kinesin expression in cultured hippocampal neurons using antisense oligonucleotides. *J. Cell Biol.* **117**, 595–606 (1992).
22. Okada, Y., Yamazaki, H., Sekine-Aizawa, Y. & Hirokawa, N. The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. *Cell* **81**, 769–780 (1995).
23. Otsuka, A. J. *et al.* The *C. elegans unc-104* gene encodes a putative kinesin heavy chain-like protein. *Neuron* **6**, 113–122 (1991).
24. Potempska, A., Styles, J., Mehta, P., Kim, K. S. & Miller, D. L. Purification and tissue level of the β -amyloid peptide precursor of rat brain. *J. Biol. Chem.* **266**, 8464–8469 (1991).
25. Hollenbeck, P. J. The distribution, abundance and subcellular localization of kinesin. *J. Cell Biol.* **108**, 2335–2342 (1989).
26. Xia, W. *et al.* Presenilin complexes with the C-terminal fragments of amyloid precursor protein at the sites of amyloid β -protein generation. *Proc. Natl Acad. Sci. USA* **97**, 9299–9304 (2000).
27. Bowman, A. B. *et al.* Kinesin-dependent axonal transport is mediated by the Sunday driver (SYD) protein. *Cell* **103**, 583–594 (2000).
28. Verhey, K. J. *et al.* Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules. *J. Cell Biol.* **152**, 959–970 (2001).
29. Kim, S. H., Lah, J. J., Thinakaran, G., Levey, A. & Sisodia, S. S. Subcellular localization of presenilins: association with a unique membrane pool in cultured cells. *Neurobiol. Dis.* **7**, 99–117 (2000).
30. Cao, X. & Sudhof, T. C. A transcriptionally active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science* **293**, 115–120 (2001).

Supplementary Information accompanies the paper on Nature's website (<http://www.nature.com>).

Acknowledgements

We thank G. Stokin for the Kif5b antibody, S. Brady for the 63–90 Klc antibody, S. Sisodia for providing the Ps1, Aplp1 and Aplp2 antibodies, and Merck for the APP-null mice. We thank D. Cleveland for discussions, and S. Emr for use of the deconvolution microscope. This work is supported by a grant from the National Institutes for Health. L.S.B.G. is an investigator of the Howard Hughes Medical Institute.

Correspondence and requests for materials should be addressed to L.S.B.G. (e-mail: lgoldstein@ucsd.edu).

Group A *Streptococcus* tissue invasion by CD44-mediated cell signalling

Colette Cywes* & Michael R. Wessels*†

* Channing Laboratory, Brigham and Women's Hospital and † Division of Infectious Diseases, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA

Streptococcus pyogenes (also known as group A *Streptococcus*, GAS), the agent of streptococcal sore throat and invasive soft-tissue infections, attaches to human pharyngeal or skin epithelial cells through specific recognition of its hyaluronic acid capsular polysaccharide by the hyaluronic-acid-binding protein CD44 (refs 1, 2). Because ligation of CD44 by hyaluronic acid can induce epithelial cell movement on extracellular matrix^{3–5}, we investigated whether molecular mimicry by the GAS hyaluronic acid capsule might induce similar cellular responses. Here we show that CD44-dependent GAS binding to polarized monolayers of human keratinocytes induced marked cytoskeletal rearrangements manifested by membrane ruffling and disruption of intercellular junctions. Transduction of the signal induced by GAS binding to CD44 on the keratinocyte surface involved Rac1 and the cytoskeleton linker protein ezrin, as well as tyrosine phosphorylation of cellular proteins. Studies of bacterial translocation in two models of human skin indicated that cell signalling triggered by interaction of the GAS capsule with CD44 opened intercellular junctions and promoted tissue penetration by GAS through a paracellular route. These results support a model of

host cytoskeleton manipulation and tissue invasion by an extracellular bacterial pathogen.

To investigate the ability of GAS to affect host epithelial cell biology, we studied the interaction of GAS with human keratinocytes—the predominant cell type in skin and the pharyngeal epithelium. Keratinocytes were grown to confluence on porous membrane supports under conditions that induced both the physiological apical–basal polarization of the epithelial cells and the formation of intercellular junctions^{6,7}. These confluent, differentiated cell cultures are referred to hereafter as monolayers, although microscopy showed partial overlap of adjacent cells so that the thickness of the layer in an individual well varied from 1 to 3 cells (see Fig. 1). Monolayers were inoculated by placement of a suspension of GAS on the apical surface of the cells. Transmission electron microscopy images showed membrane projections from keratinocytes that were exposed to wild-type GAS strain 950771, but not from cells exposed to an isogenic acapsular mutant, strain 188 (Fig. 1a). Similar membrane ruffles or lamellipodia have been observed in response to localized application of hyaluronic acid to the surface of tumour cells as a consequence of CD44-mediated cell signalling⁴.

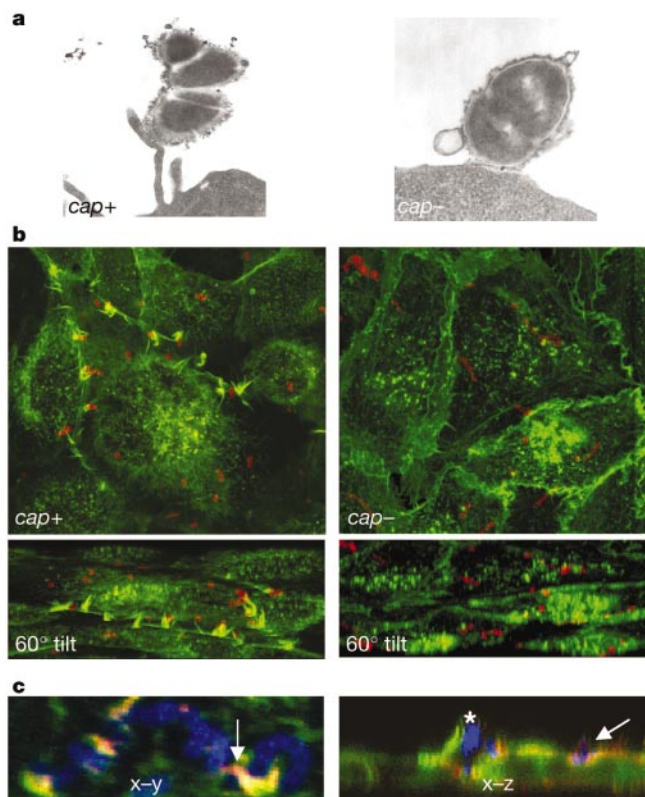


Figure 1 Induction of lamellipodia formation by GAS binding to keratinocytes. **a**, Transmission electron microscopy images showing binding of wild-type GAS (*cap*+) to membrane projections or lamellipodia of keratinocytes. Binding of the acapsular mutant strain (*cap*–) is not associated with lamellipodia formation (original magnification, 30,000 $\times$). **b**, Confocal microscopy images of GAS (red) interaction with polarized keratinocyte monolayers. Wild-type (*cap*+), but not acapsular (*cap*–), GAS are closely associated with actin (labelled green) filament projections, seen in the 60° tilt view to extend up from the plane of the monolayer (original magnification, 100 $\times$). **c**, Confocal microscopy images of interaction of wild-type GAS (blue) with polarized keratinocyte monolayers. Co-localization of actin (green) and CD44 (red) appears as yellow staining. CD44 is seen along intercellular junctions and at the tips of actin filament projections in close association with GAS (arrow in *x*–*y* panel, original magnification, 200 $\times$). The vertical section (*x*–*z* panel, original magnification, 150 $\times$) demonstrates association of GAS with CD44 on the keratinocyte surface (arrow), and lifting of the edge of a cell with closely associated GAS seen within the intercellular gap (asterisk).

In confocal microscopy images of monolayers exposed to wild-type GAS, we observed actin filament projections in close association with the bacteria (Fig. 1b). Triple-label experiments in which CD44 was visualized with specific antibody demonstrated that CD44 was distributed along intercellular junctions and at the tips of the actin filament projections, immediately adjacent to bound GAS (Fig. 1c). These microfilament projections were not observed in keratinocytes that were pretreated with anti-CD44 monoclonal antibody IM7.8.1 before inoculation with wild-type GAS, nor in untreated keratinocytes exposed to acapsular GAS. Furthermore, cell-bound acapsular bacteria were not colocalized with CD44.

In tumour cells, transduction of the extracellular event of hyaluronic acid binding to CD44 to the intracellular movement of actin filaments occurs through activation of Rac1, a member of the Rho family of small GTPases^{4,8}. To determine whether Rac1 was involved in CD44-mediated cell signalling induced by GAS, we transfected keratinocytes with N17Rac1 (dominant negative Rac1). Binding of wild-type GAS to cells transfected with N17Rac1 failed to induce formation of lamellipodia, although typical lamellipodia were induced by interaction of GAS with adjacent untransfected cells in the same keratinocyte monolayer (Fig. 2). Transfection of keratinocytes with constitutively active L61Rac1 resulted in the formation of lamellipodia in uninoculated keratinocytes. Inoculation of these cells with wild-type GAS increased the amount of lamellipodia, and wild-type GAS was observed in close association with the lamellipodia. However, binding of acapsular GAS to keratinocytes transfected with L61Rac1 was not preferentially localized to lamellipodia, but rather occurred equally over regions of the cell that were devoid of membrane projections. These results demonstrate an absolute requirement for active Rac1 in the cytoskeletal rearrangements induced by wild-type GAS.

CD44-mediated cell signalling to the cytoskeleton in other systems additionally involves ezrin, a protein of the ezrin–radixin–moesin (ERM) family, which is thought to physically link the cytoplasmic domain of CD44 to the actin cytoskeleton on activation^{9–11}. In keratinocytes that were exposed to wild-type GAS, confocal microscopy demonstrated colocalization of Rac1 and ezrin precisely at the point of attachment of GAS to the keratinocyte surface (Supplementary Information Fig. 1a, b). By contrast, binding of acapsular GAS to keratinocytes was not

associated with co-localization of Rac1 and ezrin with the bound bacteria. These results support the hypothesis that binding of the GAS hyaluronic acid capsule to keratinocyte CD44 triggers the local activation of Rac1 and association of the actin linker protein ezrin with the cytoplasmic domain of CD44. This process physically couples the keratinocyte cell membrane, by means of CD44 and ezrin, to the actin cytoskeleton. Thus, Rac1-mediated actin polymerization could result in local movement of the cell membrane to form lamellipodia and, potentially, to open intercellular junctions with neighbouring cells (see below).

Ligand binding to CD44 is expected to activate cellular protein tyrosine kinases as part of the downstream intracellular signalling cascade^{5,12}. Consistent with this model, at the site of attachment of wild-type, but not acapsular, GAS to the keratinocyte surface, confocal microscopy revealed co-localization of antibody to tyrosine-phosphorylated protein(s) with CD44 (Supplementary Information Fig. 1c). We also found that incubation of keratinocytes with the protein tyrosine kinase inhibitor, genistein, inhibited formation of lamellipodia in response to GAS, evidence that tyrosine phosphorylation of an as yet unidentified protein may serve to couple CD44 signalling to Rac1 activation.

Scanning electron microscopy and confocal microscopy images of keratinocyte monolayers that were exposed to GAS showed not only evidence of localized membrane projections, but also lifting of the margin of the keratinocyte near associated GAS, with curling back of the cell membrane from its contacts with the substratum and with the neighbouring cell (Fig. 3a). Similar morphological changes have been observed in epithelial cells subjected to localized application of hyaluronic acid or to microinjection with constitutively active Rac1 (refs 4, 11). In several instances GAS organisms were observed, below the surface of the monolayer, within the intercellular breach (see, for example, Fig. 1c). Such disruptions of intercellular junctions were not observed in keratinocytes that were exposed to acapsular GAS. We investigated whether GAS-induced disruption of intercellular junctions was associated with changes in distribution of the tight junction protein, zonula occludens-1 (ZO-1) or of E-cadherin—a member of the cadherin family of transmembrane proteins that mediate cell–cell adhesion through homophilic interaction of their extracellular domains on adjacent cells. Immunofluorescent staining demonstrated marked

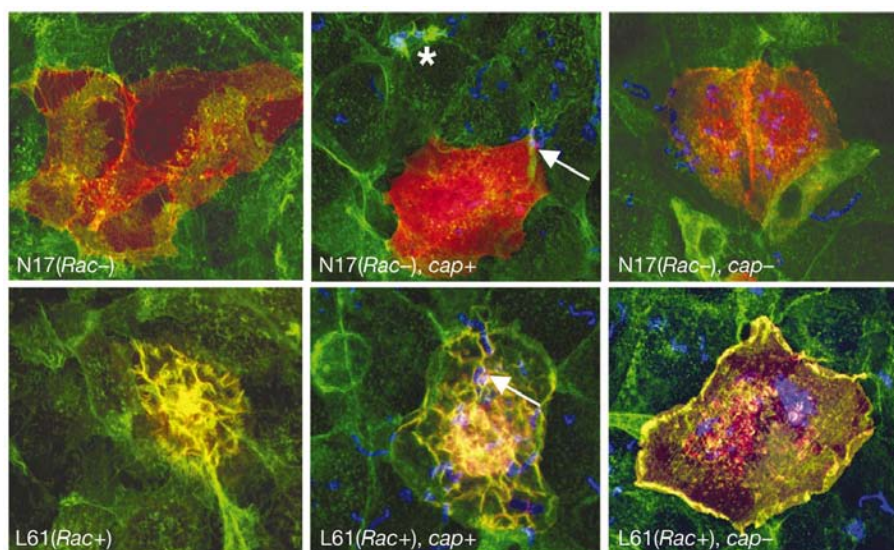


Figure 2 Confocal microscopy images of GAS interaction with keratinocytes expressing dominant negative (N17) or constitutively active (L61) Rac1. Samples were stained with antibody to the Myc tag (red) to identify transfected cells, with phalloidin (green) to label the cytoskeleton and with antibody to identify GAS (blue). Wild-type GAS (*cap+*) failed to trigger lamellipodia formation in cells transfected with N17Rac (arrow in the middle panel

of the top row), although GAS organisms were associated with lamellipodia in untransfected cells in the same monolayer (asterisk). Cells transfected with L61Rac produced abundant lamellipodia that co-localized with wild-type GAS (arrow in the middle panel of the bottom row), but not with acapsular GAS (*cap-*; bottom right panel (original magnification, 100 $\times$).

loss of both ZO-1 (Fig. 3b) and E-cadherin (not shown) from intercellular junctions after exposure of keratinocytes to wild-type, but not acapsular, GAS. This redistribution was prevented by pretreatment of the keratinocytes with genistein, implicating tyrosine kinase(s) in the process. These findings provide further evidence that interaction of the GAS hyaluronic acid capsule with CD44 results in Rac1-dependent disruption of intercellular junctions. Loss of integrity of intercellular junctions is expected to reduce the barrier function of the monolayer, as reflected by its ability to prevent the passage of small molecules such as sodium fluorescein. Infection of monolayers with the encapsulated wild-type strain resulted in an increase in permeability to sodium fluorescein (from less than 3% to greater than 30%), and a corresponding 33% decrease in transepithelial electrical resistance after 6 h of exposure. However, infection with the acapsular mutant strain resulted in no significant change in the barrier function of the monolayer.

The finding that GAS binding to CD44 induced destabilization and opening of intercellular junctions suggested that these defects in epithelial integrity might facilitate translocation of the bacteria through the epithelium. As a model of the movement of GAS from the pharyngeal mucosa or skin surface into underlying tissue, we studied GAS translocation *in vitro* through keratinocyte monolayers. Two hours after inoculation onto the surface of a confluent keratinocyte monolayer, wild-type GAS could be detected in the

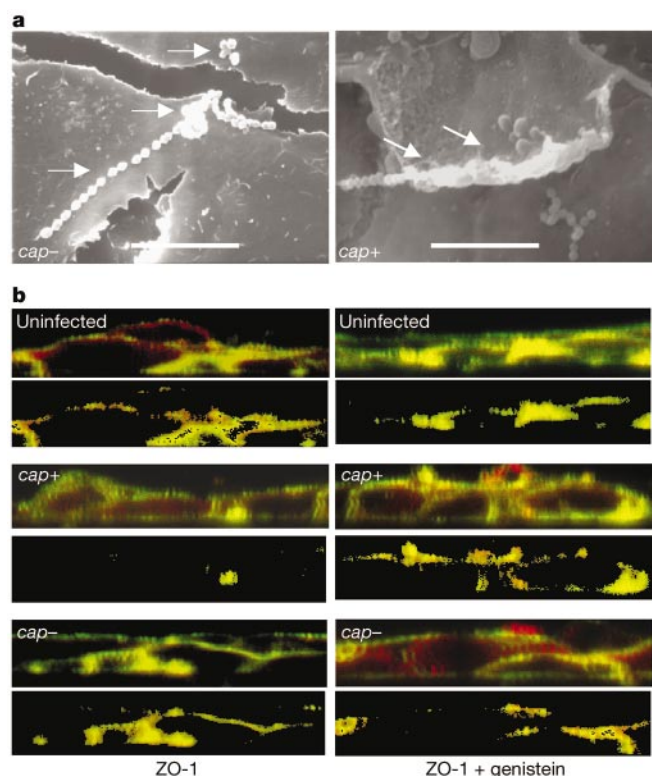


Figure 3 GAS-induced disruption of intercellular junctions. **a**, Scanning electron microscopy images of the interaction of GAS with polarized keratinocyte monolayers. Cells inoculated with wild-type strain (*cap*+), but not the acapsular strain (*cap*-), exhibit membrane ruffling and lifting and curling back of the edge of the cell around the associated GAS. The arrows indicate GAS. Scale bar, 10 μ m. **b**, Confocal microscopy images demonstrating loss of ZO-1 from intercellular junctions on exposure of keratinocytes to wild-type (*cap*+), but not acapsular (*cap*-), GAS. Samples were stained with antibody to ZO-1 (red) and with phalloidin (green) to label the cytoskeleton. Images represent vertical sections (*x-z* plane) through keratinocyte monolayers. The bottom image of each pair shows co-localization of ZO-1 and actin (indicated by yellow staining). Pretreatment with genistein prevented redistribution of ZO-1 on exposure to GAS (original magnification, 100 $\times$).

culture medium below the membrane support, indicating migration of the bacteria through the monolayer. At each of several intervals after inoculation, approximately 20–100 times as many GAS colony-forming units were recovered below monolayers inoculated with wild-type GAS compared with those inoculated with the acapsular mutant strain (Fig. 4a). A comparable difference in efficiency of translocation between wild-type and acapsular GAS was observed in separate experiments using monolayers of primary human keratinocytes (data not shown). Compared to wild-type GAS strain 950771, the efficiency of translocation was similar for wild-type GAS strains 87-282, a highly encapsulated M-type 18 strain, and DLS003, a poorly encapsulated M-type 3 strain (data not

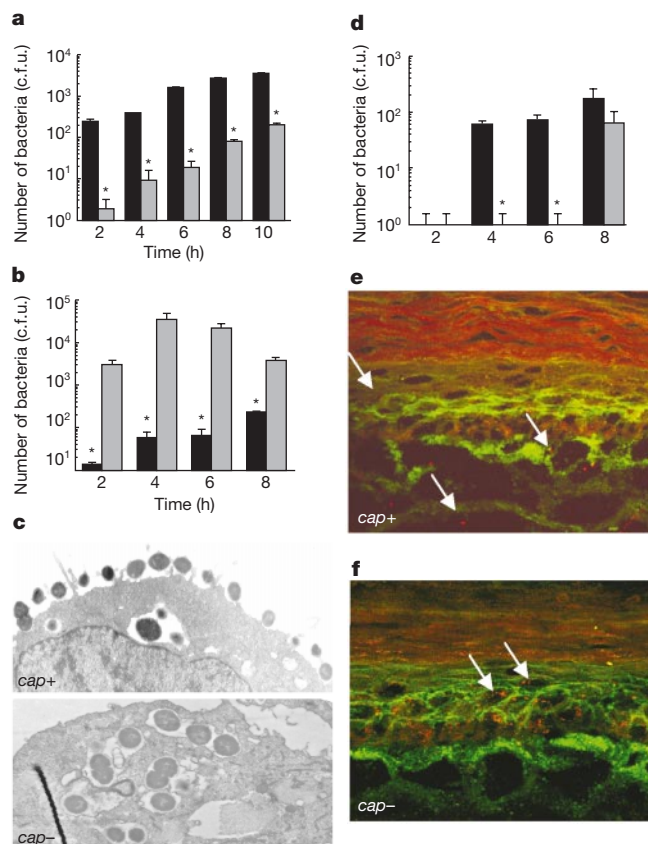


Figure 4 GAS translocation across model epithelia. **a**, Translocation of GAS through polarized keratinocyte monolayers. Data represent mean number of colony-forming units (c.f.u.) recovered from the medium beneath the monolayer at indicated times after inoculation of the apical surface with wild-type (black bars) or acapsular (grey bars) GAS. Asterisk, $P = 0.0002$ for comparison with the wild-type strain. **b**, Internalization of GAS by keratinocytes in a polarized monolayer. At indicated time points after inoculation of the apical surface of the monolayer, extracellular GAS was killed by addition of penicillin and gentamicin, and intracellular GAS was recovered after lysis of the keratinocytes. Data represent mean c.f.u. of intracellular GAS recovered from monolayers inoculated with wild-type (black bars) or acapsular (grey bars) GAS. Asterisk, $P = 0.0002$ for comparison with acapsular strain. **c**, Transmission electron microscopy images show wild-type GAS (*cap*+) associated with lamellipodia on the keratinocyte surface and a few organisms in spacious vacuoles within the cells; acapsular GAS (*cap*-) are visualized in large numbers intracellularly in membrane-bound vacuoles (original magnification, 6,500 $\times$). **d**, Translocation of GAS through human skin equivalent. Data represent mean number of c.f.u. of GAS recovered from beneath a sample of human skin equivalent (see text) at various times after inoculation of the epidermal surface with wild-type (black bars) or acapsular (grey bars) GAS. Asterisk, $P = 0.008$ for comparison with the wild-type strain. **e**, **f**, Confocal microscopy images of tissue sections of human skin equivalent 6 h after inoculation with wild-type (*cap*+) or acapsular (*cap*-) GAS. GAS organisms (indicated with arrows) are stained red and keratinocyte actin is stained green (original magnification, 100 $\times$).

shown). Translocation of wild-type GAS was reduced by 78% at 1 h in experiments in which the keratinocyte monolayers were pretreated with monoclonal antibody IM7.8.1 to block interaction of the GAS capsule with CD44. Similarly, in the presence of 250 μ M genistein, the earliest detectable translocation of wild-type GAS, was delayed from 30 min to 1 h, and the maximum number of translocated GAS at 4 h was reduced by approximately 80% (data not shown).

Transmission electron microscopy showed wild-type GAS predominantly at extracellular sites (Fig. 4c). Organisms were seen on the apical surface of the cells in association with lamellipodia and in intercellular spaces. By contrast, organisms of the acapsular mutant strain that were visualized on the surface of keratinocytes appeared to be tightly apposed to the keratinocyte cell membrane and not associated with lamellipodia; furthermore, many acapsular bacteria were observed within keratinocytes in membrane-bound vacuoles (Fig. 4c). These results were confirmed by quantitative cultures of organisms recovered from cell lysates of infected keratinocytes. At 2 h after inoculation of the keratinocyte monolayer, approximately 200-fold more intracellular GAS organisms were recovered from monolayers infected with the acapsular mutant than from monolayers infected with the wild-type strain. Similar results were obtained in studies of monolayers infected for 4, 6 or 8 h (Fig. 4b). Thus, the efficiency of translocation through the monolayer was inversely related to entry of the bacteria into the keratinocytes. Trypan blue staining did not reveal large cytopathic changes in the keratinocytes during the period of the translocation assays. Furthermore, in assays measuring release of lactate dehydrogenase as a marker of cell injury, we found greater amounts of lactate dehydrogenase released from keratinocytes that were exposed to acapsular compared with wild-type GAS (C.C. and M.R.W., unpublished observations). Therefore, the increased translocation of wild-type compared with acapsular GAS cannot be attributed to a greater cytotoxic effect by the wild-type bacteria.

To examine GAS translocation in a model system similar to normal human skin, we used pseudo-organ cultures of human skin equivalent^{13,14}. Histology of the human skin equivalent is very similar to that of normal human skin: a differentiated, stratified squamous epithelium that consists of multiple cell layers. As intact human skin is highly resistant to GAS infection, we simulated minor trauma to the superficial epidermis by abrading the surface of the skin equivalent with a cytology brush to introduce breaks in the stratum corneum. GAS organisms were inoculated onto the surface of abraded skin equivalent and translocation through the tissue was monitored at intervals by culture of GAS from the basal surface of the skin equivalent. Wild-type GAS was detected from the basal surface of the skin equivalent 4 h after surface inoculation, whereas acapsular GAS was not detected until 8 h after inoculation (Fig. 4d). Examination of histological sections by confocal microscopy revealed wild-type GAS in intercellular spaces throughout the epidermis and dermis, whereas acapsular GAS organisms were visualized primarily in the superficial layers of the epidermis, often within keratinocytes (Fig. 4e, f). Thus, capsule-deficient organisms fail to translocate efficiently, but rather remained trapped within keratinocytes in the superficial epidermis. These findings extend similar results obtained using keratinocyte monolayers to a system with important features of normal human skin.

Our results provide evidence for the following mechanism in bacterial pathogenesis: binding of the GAS hyaluronic acid capsule to CD44 on epithelial cells triggers cytoskeletal rearrangements that open intercellular junctions, thereby facilitating paracellular translocation of the organisms from the epithelial surface into underlying tissues. The CD44-mediated cell signalling events involve tyrosine phosphorylation of downstream effector proteins and the direct or indirect interaction of Rac1 and ezrin with the cytoplasmic domain of CD44. Organisms such as *Salmonella typhimurium* and *Shigella* spp. also elicit epithelial cell cytoskeletal rearrangements

and membrane ruffling through activation of Rho family GTPases^{15,16}. However, an important difference between GAS and Gram-negative enteric pathogens is the consequence of cytoskeletal rearrangements induced by bacterial contact with the host epithelial cell. In the epithelial cell response to *Salmonella* or *Shigella* spp., extension and subsequent fusion of lamellipodia entraps the bacteria and results in their internalization. By contrast, although interaction of GAS with CD44 produces marked membrane ruffling, subsequent internalization of wild-type (encapsulated) GAS is relatively inefficient, and most of the cell-associated bacteria remain extracellular. The predominant effect of CD44-mediated cytoskeletal rearrangements seems to be disruption of intercellular junctions to permit the passage between cells of GAS, which remain extracellular as they penetrate the epithelium. Thus, the hyaluronic acid capsule not only facilitates tissue invasion by disrupting intercellular junctions, but also by preventing internalization and trapping of the organisms within epithelial cells.

As the GAS strains that cause severe invasive infections such as necrotizing fasciitis do so only rarely—whereas pharyngitis caused by the same strains is common—it is clear that host factors are critical determinants in invasive disease. Nevertheless, the experimental observation that capsule-deficient mutants are avirulent in a mouse model of necrotizing fasciitis implies that capsule may be one of perhaps several bacterial factors that are required for invasive infection to occur¹⁷. Our findings support paracellular translocation as the mechanism of tissue invasion in such infections. Results of this study define a previously unknown virulence mechanism of a prototypic extracellular bacterial pathogen. □

Methods

Bacterial strains and polarized keratinocyte monolayers

The GAS strains that we used were 950771, an M-type 3 strain originally isolated from a child with necrotizing fasciitis, and 188, an isogenic acapsular mutant derived from 950771 (ref. 17). Bacteria were cultured in Todd Hewitt broth (Difco) to early exponential phase. For preparation of polarized keratinocyte monolayers, human squamous cell carcinoma cell line SCC13, derived from cheek epidermis¹⁸, was cultured to confluence in serum-free keratinocyte medium (SFM, Gibco BRL) supplemented with 50 μ g ml⁻¹ bovine pituitary extract, 0.1 ng ml⁻¹ epidermal growth factor and 0.3 mM calcium chloride (c-SFM). Certain experiments were performed using cell monolayers prepared from OKP7 primary human keratinocytes¹⁹. Keratinocytes were seeded at 5×10^5 cells per well onto polycarbonate Transwell membrane supports (12-well plates, 3.0- μ m pore size; Costar) and cultured for 6–10 days at 37 °C with 5% CO₂ in c-SFM, with daily medium changes. Once confluent, the integrity of the monolayer was assessed routinely by measuring permeability to sodium fluorescein²⁰ and, in some experiments, by measuring transepithelial electrical resistance²¹.

Antibody labelling and confocal microscopy

Keratinocyte monolayers were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), washed, permeabilized with 0.1% Triton X-100 in PBS, then incubated with 0.5% bovine serum albumin (BSA) in PBS to block nonspecific binding sites before antibody staining. Primary antibodies included rabbit immunoglobulin- γ (IgG) antibody to GAS group A carbohydrate (ImmuCell) at 5 μ g ml⁻¹, rat monoclonal anti-mouse CD44 (clone IM7.8.1)¹ at 15 μ g ml⁻¹, goat IgG anti-ezrin at 2 μ g ml⁻¹ (Santa Cruz Biotechnology), goat IgG anti-Rac1 at 2 μ g ml⁻¹ (Santa Cruz Biotechnology), mouse anti-phosphotyrosine at 2 μ g ml⁻¹ (PY-Plus, Zymed Laboratories), rabbit anti-TJP1 at 2 μ g ml⁻¹ (Zymed), goat anti-E-cadherin at 2 μ g ml⁻¹ (Santa Cruz Biotechnology) and mouse anti-Myc monoclonal antibody 9E10 (Santa Cruz Biotechnology). Secondary antibodies included, as appropriate, Texas red- or Alexa 660-conjugated anti-rabbit IgG (Molecular Probes), Alexa 568-conjugated anti-rabbit, anti-goat, or anti-rat IgG (Molecular Probes) and fluorescein isothiocyanate-conjugated anti-goat or anti-mouse IgG (Sigma), each diluted 1:250 in 0.5% BSA/PBS. Cellular actin was labelled with 165 nm phalloidin conjugated to Oregon green 514 or to Alexa 488 (Molecular Probes).

Confocal microscopy images were collected using a BioRad scanning confocal microscope equipped with a krypton–argon laser. We processed images with BioRad Confocal Assistant and Adobe Photoshop 5.5 (Adobe Systems).

Transfection of SCC13 keratinocytes with Rac constructs

Eukaryotic expression plasmids pRK5-Myc-N17Rac1 (Myc-tagged, dominant negative Rac1) and pRK5-Myc-L61Rac1 (Myc-tagged, constitutively active Rac1) were provided by A. Hall. Polarized SCC13 keratinocyte monolayers were transiently transfected using 5 μ g of either of the two expression constructs and 30 μ l SuperFect transfection reagent (Qiagen). Twenty-four hours later the monolayers were inoculated with GAS as described below.

GAS translocation through polarized keratinocyte monolayers

Keratinocyte monolayers on Transwells were inoculated with GAS at a multiplicity of infection of 10 per keratinocyte. Monolayers were incubated at 37 °C with 5% CO₂. The keratinocyte medium in the upper chamber was replaced 2 h after adding GAS, and the Transwell inserts containing the keratinocytes were moved to new wells containing fresh medium every 2 h to prevent overgrowth of translocated bacteria. We assessed translocation by quantitative cultures of medium from the lower chamber at 2 h intervals.

Apligraf living skin equivalent (a gift from Organogenesis) was used as a model for human skin¹⁴. Samples of skin equivalent were cut into rectangular sections, each approximately 2.5 × 1.2 cm. Each section was placed on a 12 mm Transwell (3.0-μm pore size; Costar) and the Transwell was placed on Todd Hewitt agar containing 5% sheep blood. The apical surface of the skin equivalent was lightly brushed with a sterile cytology brush to abrade the stratum corneum, exposing the underlying epidermal keratinocytes. GAS was resuspended at 10⁶ colony-forming units per 10 μl⁻¹ in SFM supplemented with 0.3 mM calcium chloride, and inoculated onto the surface of the tissue. After inoculation, we incubated tissue samples at 37 °C with 5% CO₂ and no supplemental humidity. Transwells containing the inoculated tissue samples were transferred to fresh blood agar every 2 h. The blood agar plates were then incubated overnight at 37 °C for enumeration of colony-forming units representing the number of organisms emerging from the basal surface of the tissue.

Received 20 August; accepted 4 October 2001.

- Schrager, H. M., Alberti, S., Cywes, C., Dougherty, G. J. & Wessels, M. R. Hyaluronic acid capsule modulates M protein-mediated adherence and acts as a ligand for attachment of group A *Streptococcus* to CD44 on human keratinocytes. *J. Clin. Invest.* **101**, 1708–1716 (1998).
- Cywes, C., Stamenkovic, I. & Wessels, M. R. CD44 as a receptor for colonization of the pharynx by group A *Streptococcus*. *J. Clin. Invest.* **106**, 995–1002 (2000).
- Lesley, F., Hyman, R. & Kincade, P. W. CD44 and its interaction with extracellular matrix. *Adv. Immunol.* **54**, 271–335 (1993).
- Oliferenko, S., Kaverina, I., Small, J. V. & Huber, L. A. Hyaluronic acid (HA) binding to CD44 activates Rac1 and induces lamellipodia outgrowth. *J. Cell Biol.* **148**, 1159–1164 (2000); erratum *J. Cell Biol.* **149**, 236 (2000).
- Bourguignon, L. Y. W., Zhu, H., Shao, L. & Chen, Y.-W. CD44 interaction with c-Src kinase promotes cortactin-mediated cytoskeleton function and hyaluronic acid-dependent ovarian tumor cell migration. *J. Biol. Chem.* **276**, 7327–7336 (2001).
- Rheinwald, J. G. The role of terminal differentiation in the finite culture lifetime of the human epidermal keratinocyte. *Int. Rev. Cytol. Suppl.* **10**, 25–33 (1979).
- Rheinwald, J. G. Serial cultivation of normal human epidermal keratinocytes. *Methods Cell Biol.* **21A**, 229–254 (1980).
- Bourguignon, L. Y., Zhu, H., Shao, L. & Chen, Y. W. CD44 interaction with tiam1 promotes Rac1 signaling and hyaluronic acid-mediated breast tumor cell migration. *J. Biol. Chem.* **275**, 1829–1838 (2000).
- Tsukita, S., Oishi, K., Sato, N., Sagara, J. & Kawai, A. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J. Cell Biol.* **126**, 391–401 (1994).
- Legg, J. W. & Isacke, C. M. Identification and functional analysis of the ezrin-binding site in the hyaluronan receptor, CD44. *Curr. Biol.* **8**, 705–708 (1998).
- Hall, A. Rho GTPases and the actin cytoskeleton. *Science* **279**, 509–514 (1998).
- Taher, T. E. et al. Signaling through CD44 is mediated by tyrosine kinases. Association with p56lck in T lymphocytes. *J. Biol. Chem.* **271**, 2863–2867 (1996).
- Parenteau, N. L., Bilbo, P., Nolte, C. J., Mason, V. S. & Rosenberg, M. The organotypic culture of human skin keratinocytes and fibroblasts to achieve form and function. *Cytotechnology* **9**, 163–171 (1992).
- Sabolinski, M. L., Alvarez, O., Auletta, M., Mulder, G. & Parenteau, N. L. Cultured skin as a 'smart material' for healing wounds: experience in venous ulcers. *Biomaterials* **17**, 311–320 (1996).
- Finlay, B. B. & Cossart, P. Exploitation of mammalian host cell functions by bacterial pathogens. *Science* **276**, 718–725 (1997).
- McCallum, S. J. & Theriot, J. A. In *Cellular Microbiology* (eds Cossart, P., Boquet, P., Normark, S. & Rappuoli, R.) 171–191 (ASM, Washington DC, 2000).
- Ashbaugh, C. D., Warren, H. B., Carey, V. J. & Wessels, M. R. Molecular analysis of the role of the group A streptococcal cysteine protease, hyaluronic acid capsule, and M protein in a murine model of human invasive soft-tissue infection. *J. Clin. Invest.* **102**, 550–560 (1998).
- Cline, P. R. & Rice, R. H. Modulation of involucrin and envelope competence in human keratinocytes by hydrocortisone, retinyl acetate, and growth arrest. *Cancer Res.* **43**, 3203–3207 (1983).
- Crowe, D. L., Hu, L., Gudas, L. J. & Rheinwald, J. G. Variable expression of retinoic acid receptor (RARβ) mRNA in human oral and epidermal keratinocytes; relation to keratin 19 expression and keratinization potential. *Differentiation* **48**, 199–208 (1991).
- Tchao, R. In *Alternative Methods in Toxicology* (ed. Goldberg, A. M.) 271–283 (Ann Liebert, New York, 1988).
- McNamara, B. P. et al. Translocated EspF protein from enteropathogenic *Escherichia coli* disrupts host intestinal barrier function. *J. Clin. Invest.* **107**, 621–629 (2001).

Supplementary Information accompanies the paper on Nature's website (<http://www.nature.com>).

Acknowledgements

We thank E. Meluleni for preparation of the histology specimens; M. Lowe for assistance with the confocal microscopy; and R. Stearns for assistance with scanning electron microscopy. This work was supported by the National Institutes of Health.

Correspondence and requests for materials should be addressed to M.R.W. (e-mail: mwessels@channing.harvard.edu).

The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol

Yihong Ye*, Hemmo H. Meyer† & Tom A. Rapoport*

* Howard Hughes Medical Institute and Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, USA

† Department of Cell Biology, Yale Medical School, 333 Cedar Street, SHM C432, New Haven, Connecticut 06520, USA

In eukaryotic cells, incorrectly folded proteins in the endoplasmic reticulum (ER) are exported into the cytosol and degraded by the proteasome¹. This pathway is co-opted by some viruses. For example, the US11 protein of the human cytomegalovirus targets the major histocompatibility complex class I heavy chain for cytosolic degradation². How proteins are extracted from the ER membrane is unknown. In bacteria and mitochondria, members of the AAA ATPase family are involved in extracting and degrading membrane proteins^{3,4}. Here we demonstrate that another member of this family, Cdc48 in yeast and p97 in mammals, is required for the export of ER proteins into the cytosol. Whereas Cdc48/p97 was previously known to function in a complex with the cofactor p47 (ref. 5) in membrane fusion^{6–8}, we demonstrate that its role in ER protein export requires the interacting partners Ufd1 and Npl4. The AAA ATPase interacts with substrates at the ER membrane and is needed to release them as polyubiquitinated species into the cytosol. We propose that the Cdc48/p97–Ufd1–Npl4 complex extracts proteins from the ER membrane for cytosolic degradation.

We proposed that Cdc48/p97 may have a role in ER protein degradation on the basis of several pieces of indirect evidence. First, Cdc48 binds to Ufd1, Ufd2 and Ufd3 (refs 9–11), which are involved in the degradation of ubiquitin-fusion proteins¹². Second, a complex of Cdc48, Ufd1 and Npl4 (a protein originally identified in a screen for nuclear transport defects¹³) functions in the proteasome-dependent cleavage of ER membrane proteins that serve as the precursors to two transcription factors (Spt23 and Mga2)¹⁴. Third, both mammalian p97 and yeast Cdc48 interact with the proteasome and a degradation substrate^{15,16}. Finally, a large fraction of both Cdc48 and p97 is associated with ER membrane (ref. 6 and Y.Y., unpublished results).

To test directly the involvement of Cdc48 in ER protein degradation, we analysed the stability of misfolded ER proteins in *CDC48* mutants of *Saccharomyces cerevisiae*. As a membrane protein substrate we choose H–2K^b, a major histocompatibility complex (MHC) class I heavy chain that in yeast is quickly degraded in a proteasome-dependent manner¹⁷. Wild-type or *cdc48-1* mutant cells were pulse-labelled with [³⁵S]methionine at a non-permissive temperature and incubated with unlabelled methionine for different time periods. Cell lysates were subjected to immunoprecipitation with antibodies to H–2K^b. The results showed that H–2K^b was much more stable in *cdc48-1* mutant than in wild-type cells (Fig. 1a). A significant stabilization of H–2K^b was seen with another *CDC48* mutant (*cdc48-3*), even at permissive temperature (Fig. 1b).

We tested the misfolded luminal protein CPY*, an aberrant form of carboxypeptidase Y (CPY), which is degraded by the ubiquitin-proteasome pathway¹⁸. The stability of CPY* was analysed by immunoblotting after inhibition of protein synthesis with cycloheximide. Whereas CPY* was rapidly degraded in wild-type cells at both 30 °C and 23 °C, the protein was significantly more stable in *cdc48-1* cells (Fig. 1c), particularly at 23 °C—the restrictive temperature for the mutant. The half-life of CPY* was also increased in

cdc48-3 mutant cells (Fig. 1d). Together, these results show that the degradation of both membrane and soluble ER proteins depends on the function of Cdc48.

Next we tested the role of Ufd1 and Npl4, two known interaction partners of Cdc48 (refs 14, 19), in the degradation of ER proteins. In the *ufd1-1* mutant both H-2K^b and CPY* were significantly stabilized (Fig. 2a, c). Similar results were obtained with *NPL4* mutants even at permissive temperature (Fig. 2b; also compare upper and middle row of panels d, e). When mutant cells expressed the wild-type Npl4 protein, rapid degradation of CPY* was restored (Fig. 2d, e, lower rows). These data indicate that Ufd1 and Npl4 also have a role in ER protein degradation. Other Ufd proteins (Ufd2, Ufd4 and Ufd5) do not seem to be involved, as deletion mutants did not show any defects in ER protein degradation (Supplementary Information Fig. 1).

In *CDC48*, *UFD1* and *NPL4* mutants, stabilized CPY* appears to remain inside the ER as indicated by the fact that it remained associated with the membrane and protected from added protease (Supplementary Information Fig. 2). As Cdc48 interacts with Ufd1 and Npl4 (refs 14, 19), these data suggest that the complex is required for the export of misfolded ER substrates. Consistent with the observation that the accumulation of misfolded proteins in the ER generally leads to the induction of the unfolded protein response (UPR)¹⁷, β -galactosidase under the control of a UPR-element-containing promoter was expressed in *cdc48-3*, *ufd1-1* and *npl4-2* mutants, but not in the corresponding wild-type strains (Fig. 2f).

Next we tested whether p97, the mammalian homologue of yeast Cdc48, has a similar role in the export of ER proteins. Retrograde translocation of MHC class I heavy chains, induced by the cyto-

megalovirus protein US11, was studied in permeabilized astrocytoma cells in the presence of a proteasome inhibitor to prevent subsequent heavy chain degradation in the cytosol^{2,20}.

To interfere with the function of p97, we generated a dominant negative mutant similar to one used for the trypanosomal homologue²¹. An ATP hydrolysis-defective mutant was constructed by changing the glutamic acid residues at positions 305 and 578 in rat p97—located within the Walker B motifs of the two ATPase domains—to glutamine residues (QQ mutant). Wild-type p97 and the QQ mutant were expressed as His-tagged proteins in *Escherichia coli*, and purified. As expected, the ATPase activity of the mutant p97 was at least tenfold lower than that of wild-type p97, but the interactions with Npl4/Ufd1 and p47 were maintained (Supplementary Information Fig. 3).

Astrocytoma cells expressing US11 were pulse-labelled with [³⁵S]-methionine and permeabilized. Either wild-type or mutant p97 was added at about threefold excess over endogenous protein (Supplementary Information Fig. 4). After a chase incubation, the samples were either analysed directly by immunoprecipitation with anti-heavy-chain antibody, or first fractionated into a pellet fraction (containing the ER membranes) and a supernatant fraction (containing cytosol) before analysis (Fig. 3a). In the absence of added p97, MHC heavy chains are exported from the ER and deglycosylated in the cytosol^{2,20}. Accordingly, deglycosylated chains appeared during the chase (lane 2) and fractionated with the cytosol (lane 4),

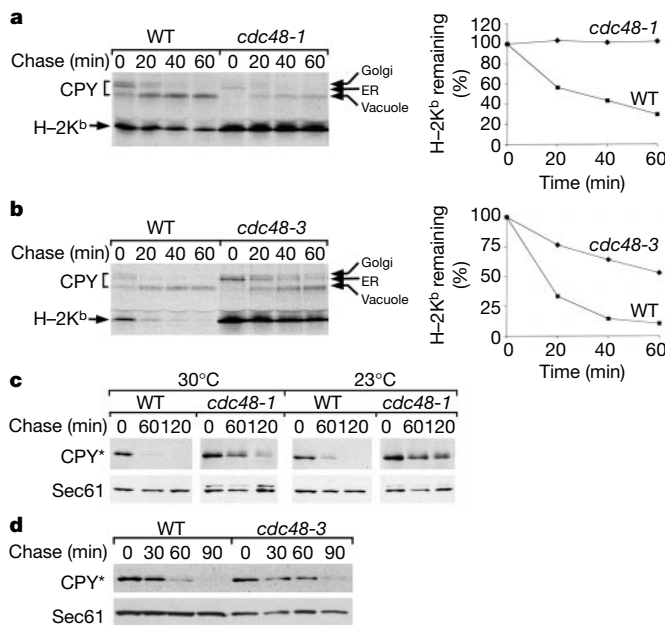


Figure 1 Cdc48 is required for ER protein degradation in *S. cerevisiae*. **a**, Stability of the heavy chain H-2K^b analysed in pulse-chase experiments at 23 °C for wild-type (WT) and *cdc48-1* yeast cells. Transport of carboxypeptidase Y (CPY) from the ER via the Golgi to the vacuole¹⁷ was not affected in the mutant (the different forms of CPY are indicated). The graph shows quantification of the experiment. **b**, Stability of H-2K^b analysed in WT and *cdc48-3* cells at 30 °C. The graph shows the mean of two experiments. **c**, Stability of CPY* analysed in WT and *cdc48-1* cells at the indicated temperatures by immunoblotting after addition of cycloheximide. Immunoblotting with Sec61 antibodies was used to verify equal loading. **d**, Stability of CPY* in WT and *cdc48-3* cells at 30 °C. The same results were obtained when isogenic WT and *cdc48-3* strains, generated by three rounds of backcrossing, were compared, or when oleic acid was added to the growth medium to compensate for potential defects in lipid synthesis of this mutant (not shown).

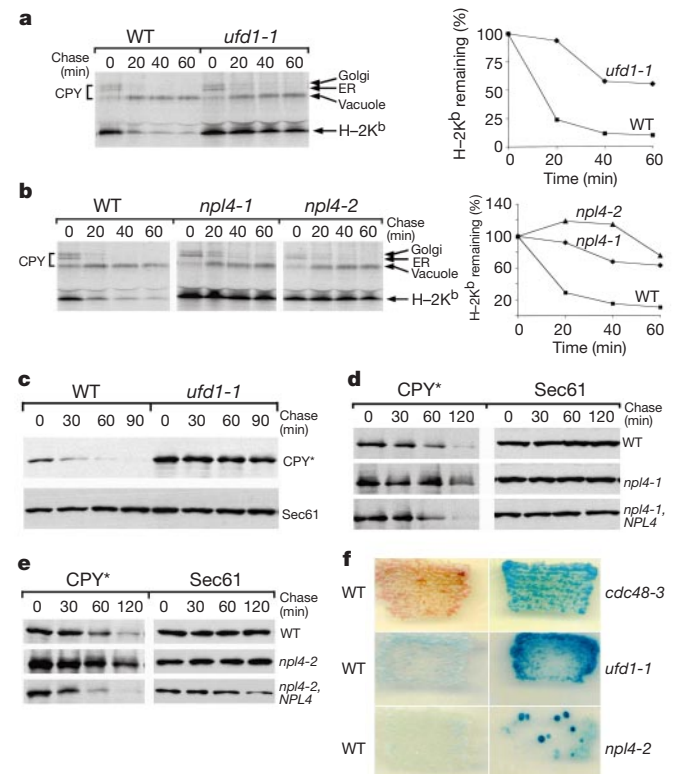


Figure 2 The Cdc48 interacting proteins Ufd1 and Npl4 are required for ER protein degradation. **a**, Stability of H-2K^b analysed by pulse-chase experiments at 25 °C in wild-type (WT) and *ufd1-1* cells. The graph shows the quantification of the experiment. **b**, Stability of H-2K^b in WT, *npl4-1* and *npl4-2* cells. The graph shows quantification of the experiment. **c**, Stability of CPY* analysed in WT and *ufd1-1* cells by immunoblotting after addition of cycloheximide at 30 °C. Immunoblotting with Sec61 antibodies served as a loading control. **d**, Stability of CPY* analysed in WT and *npl4-1* cells. Where indicated, the cells expressed the WT gene *NPL4* on a plasmid. **e**, Stability of CPY* analysed in WT and *npl4-2* cells. **f**, Expression of β -galactosidase under a UPR-element-containing promoter in mutant and corresponding WT strains, visualized by the appearance of blue.

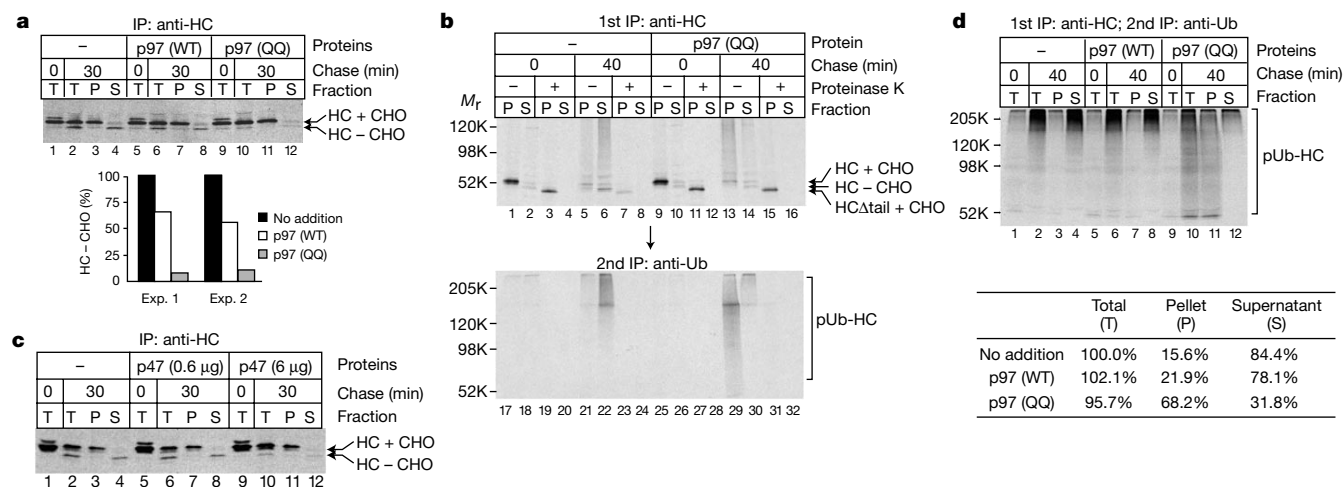


Figure 3 Mammalian p97 is required for export of MHC class I heavy chains from the ER. **a**, US11-expressing cells were labelled, permeabilized and chase-incubated with cytosol alone (-) or addition of wild-type p97 (p97 (WT)) or mutant protein p97 (QQ). They were either analysed directly (T) or first fractionated into membrane pellet (P) and supernatant (S) fractions before immunoprecipitation (IP) with heavy chain (HC) antibodies. HC + CHO and HC - CHO indicate glycosylated and deglycosylated HC, respectively. The graph shows the relative amount of deglycosylated heavy chains accumulated during the chase in two experiments. **b**, After the chase incubation as in **a**, proteinase K was added as

indicated, and the samples were subjected to immunoprecipitation with heavy chain antibodies (top panel). HCΔtail + CHO, glycosylated HC, the cytoplasmic tail of which has been cleaved off. A portion of the samples was subjected to another round of immunoprecipitation with ubiquitin (Ub) antibodies (lower panel). pUb-HC, polyubiquitinated HC. **c**, As in **a**, with the indicated amounts of p47 added. **d**, Ubiquitinated heavy chains in the membrane and supernatant fractions analysed by double immunoprecipitation as in **b**. The table shows quantification of the ubiquitinated heavy chains at the end of the chase.

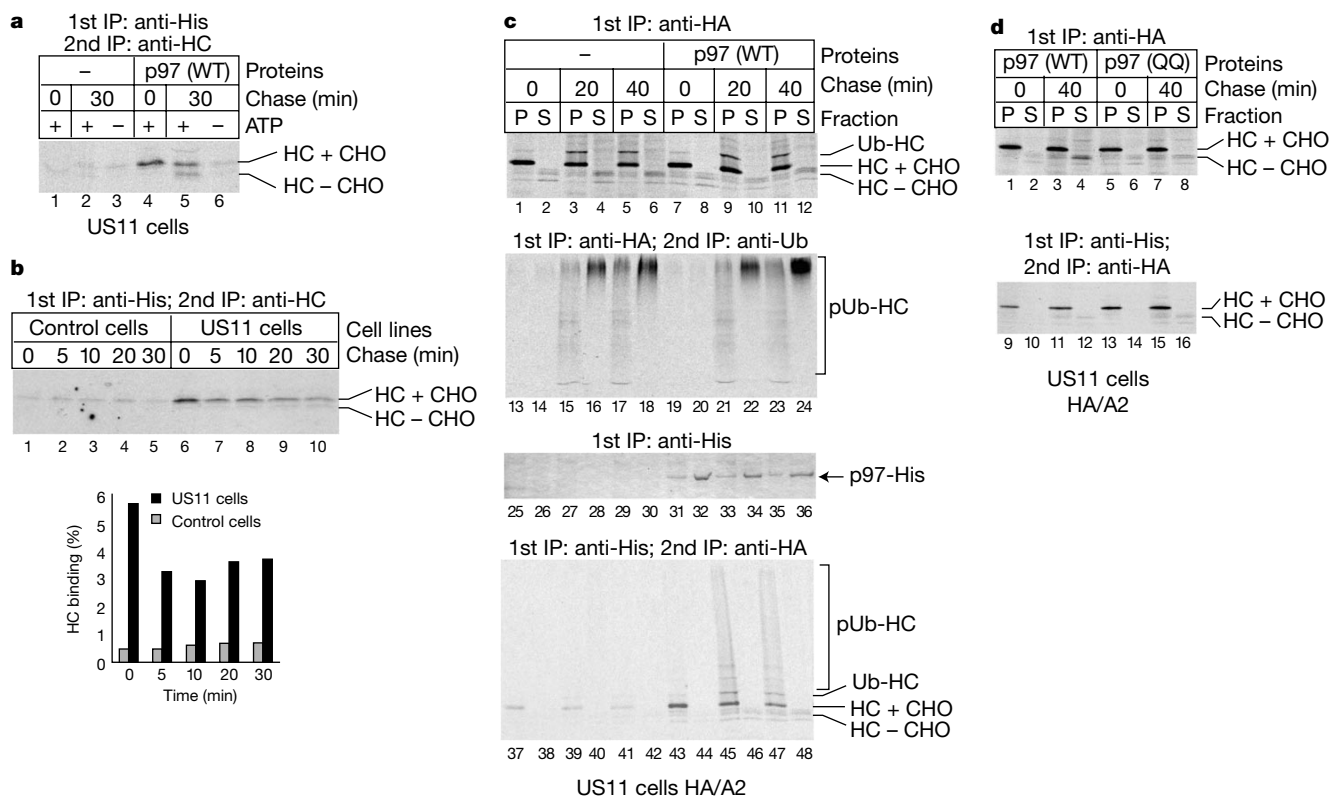


Figure 4 Association of p97 with MHC class I heavy chains. **a**, US11-expressing cells were labelled and permeabilized in the presence or absence of His-tagged wild-type p97 (p97 (WT)). Where indicated, ATP was depleted. The samples were chase-incubated for the indicated time period, solubilized and subjected to sequential immunoprecipitation (IP) with His and heavy chain (HC) antibodies. HC + CHO and HC - CHO indicate glycosylated and deglycosylated HC, respectively. **b**, As in **a**, but with both control and US11-expressing cells. The graph shows the amount of p97-associated heavy chains relative to the total amount, determined in separate immunoprecipitations (not shown). **c**, As in **a**, but with a cell line expressing both US11 and HA-tagged heavy chain (HA/A2). The samples were fractionated into a membrane pellet (P) and cytosol (S) fraction. Ten per cent of each

fraction was used for direct immunoprecipitation for heavy chains with HA antibodies (lanes 1–12). Twenty per cent of each fraction was sequentially immunoprecipitated with HA and ubiquitin (Ub) antibodies (lanes 13–24). The remainder of each fraction was immunoprecipitated with His antibodies. An aliquot was analysed by staining with Coomassie blue (lanes 25–36), and the rest was subjected to a second immunoprecipitation with HA antibodies (lanes 37–48). pUb-HC and Ub-HC indicate polyubiquitinated and mono-ubiquitinated HC, respectively. **d**, As in **c**, except that both wild-type (WT) and mutant p97 were used. Ten per cent of the sample was directly immunoprecipitated with HA antibodies (lanes 1–8). The rest of the sample was sequentially immunoprecipitated with His and HA antibodies (lanes 9–16).

whereas the glycosylated chains remained in the ER (lane 3). The addition of wild-type p97 had a moderate effect on retro-translocation of the heavy chain. On the other hand, mutant p97 strongly inhibited the export of heavy chains (Fig. 3a). These chains remained inside the ER as only their cytoplasmic tails were degraded by added protease (Fig. 3b, compare lane 15 and lane 7).

We attempted to inhibit the function of p97 in ER protein degradation by addition of p47, the cofactor required for membrane fusion⁵. This protein competes with Ufd1 and Npl4 for p97 binding¹⁹ and decreases the ATPase activity of p97 (ref. 22). We found that addition of purified recombinant p47 to permeabilized cells inhibited retro-translocation of MHC heavy chains (Fig. 3c).

Next we tested the effect of mutant p97 and of p47 on the polyubiquitination of MHC heavy chains, a process that precedes the release of the chains into the cytosol^{20,23}. The material that was immunoprecipitated with heavy chain antibodies was subjected to a second round of immunoprecipitation with ubiquitin antibodies to enrich for the small fraction of ubiquitinated heavy chains. In the absence of added p97, polyubiquitinated chains accumulated during the chase period and appeared in the cytosol fraction. Wild-type p97 had little effect on polyubiquitination and the release of these chains from the ER membrane. In contrast, the addition of mutant p97 inhibited the release of ubiquitinated chains into the cytosol but did not affect the total amount of ubiquitinated heavy chains (Fig. 3d). The heavy chains accumulating on the membrane carried fewer ubiquitin molecules than those in the cytosol (Fig. 3b, d), suggesting that long ubiquitin chains are required for release of the substrate from the membrane. As expected, the ubiquitinated chains on the membrane were sensitive to protease treatment (Fig. 3b). The release of polyubiquitinated heavy chains into the cytosol was also inhibited by the addition of p47 (Supplementary Information Fig. 5).

Finally, we investigated whether p97 associates with substrate. His-tagged p97 protein was added to labelled permeabilized cells, the membranes were solubilized in a mild detergent, and p97 was precipitated with anti-His antibodies. A significant proportion of the MHC heavy chains was co-precipitated, even at the beginning of the chase period, as demonstrated by a second immunoprecipitation (Fig. 4a, lanes 4 and 5). Co-precipitation was reduced when ATP was depleted (lane 6). In the absence of added p97 very little material was co-precipitated (lanes 1–3). Similar results were obtained with cells that express haemagglutinin (HA)-tagged MHC heavy chains, using HA antibodies in the second immunoprecipitation²⁰ (Supplementary Information Fig. 6). The association between p97 and MHC heavy chains was much more pronounced in US11-expressing cells than in control cells (Fig. 4b, lanes 6–10 compared with 1–5; see also quantification graph). Binding was not detected if p97 was added after solubilization of the membranes (Supplementary Information Fig. 7). Cell fractionation showed that p97 associated preferentially with heavy chains on the membrane (Fig. 4c, lanes 43–48), despite the fact that most of the added p97 was cytosolic (Fig. 4c, lanes 31–36). Polyubiquitinated heavy chains were much less abundant on the membrane than in the cytosol (Fig. 4c, lanes 21–24), yet only the ones on the membrane, carrying fewer ubiquitin molecules, were found in association with p97 (Fig. 4c, lanes 45 and 47). Although the ATP hydrolysis mutant of p97 did not support release of substrate into the cytosol, it did interact with heavy chains (Fig. 4d).

Taken together, our data indicate that the Cdc48/p97–Ufd1–Npl4 complex is involved in ER protein degradation in yeast and mammals. The AAA ATPase binds the substrate on the ER membrane and releases it into the cytosol on ATP hydrolysis. Compromising the function of Cdc48/p97 leads to the accumulation of substrates in the ER lumen, although a small portion is found as polyubiquitinated molecules on the membrane. Exactly how ubiquitination cooperates with Cdc48/p97-driven substrate release remains to be clarified. Although p97 can recognize ubiquitin²⁴,

we found that the ATPase was associated with both unmodified and modified MHC class I heavy chains, suggesting that p97 binds substrates either before or during polyubiquitination. On the basis of our data, we propose that Cdc48/p97 is involved in extracting proteins from the ER membrane. This function would be similar to that of other members of the AAA family of ATPases, which contain an additional protease domain (AAA proteases). FtsH in *E. coli* and AAA proteases in mitochondria appear to grab a misfolded membrane protein at one terminus and sequentially pull it out for degradation^{3,4}. The structure of p97 indicates that there are two hexameric rings stacked on top of each other with a pore in the middle²⁵. One possibility is that the Cdc48/p97 ring is bound to the ER membrane and pulls the polypeptide chain through its pore in an ATP-dependent manner. This would be analogous to the mechanism by which hexameric helicases move along single-stranded nucleic acids²⁶, or by which the hexameric ATPase rings move polypeptides into the associated proteolytic chambers of the eukaryotic proteasome or the bacterial ClpP²⁷. □

Methods

Strains and plasmids

The wild-type strains used in this study were: W303α (*MATα*, *his3-11*, *trp1-1*, *ura3-1*, *ade2-1*, *leu2-3,-112*, *can1-100*); BWG1-7a (*MATα*, *his4-519*, *ura3-52*, *ade1-100*, *leu2-3,-112*), provided by E. Johnson as an isogenic strain for *ufd1-1*; S288C (*MATα*, *ura3-52*, *leu2Δ1*, *trp1Δ63*), provided by P. Silver as an isogenic strain for *npl4-1* and *npl4-2*; and BY4742 (*MATα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*), obtained from Research Genetics as an isogenic strain for *Δufd2*, *Δufd3*, *Δufd4*, *Δufd5*. MLY1763 (*ade2-101*, *lys2-801*, *ura3-52*, *cdc48-1*), PSY2340 (*MATα*, *ura3-52*, *leu2Δ1*, *trp1Δ63*, *npl4-1*), PSY2341 (*MATα*, *ura3-52*, *leu2Δ1*, *trp1Δ63*, *npl4-2*) and the rescue plasmid pPS402 containing the *NPL4* gene were a gift from P. Silver. WPY106 (*MATα*, *ura3-52*, *leu2-3*, *cdc48-3*) was provided by W. Prinz. We backcrossed this strain three times to W303α to obtain an isogenic pair. PM373 (*MATα*, *his4-519*, *ura3-52*, *ade1-100*, *leu2-3,-112*, *ufd1-1*) was provided by E. Johnson. Strains 13888 (*MATα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*, *Δufd2*), 15063 (*MATα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*, *Δufd3*), 14859 (*MATα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*, *Δufd4*) and 13716 (*MATα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*, *Δufd5*) were obtained from Research Genetics. The pTS-A2 plasmid expressing H-2K^b was provided by H. Ploegh, and the pRS306-*prc1-1* plasmid encoding CPY* was a gift from T. Sommer. The pMCZ-Y plasmid containing the UPRE-lacZ reporter was provided by R. Schekman.

Antibodies

Antibodies to MHC class I heavy chain, ubiquitin and HA (12CA5) have been described previously²⁰, as have those to CPY¹⁷ and to Kar2 and Sec61 (ref. 28). The anti-Cdc28 antibody was a gift from N. Bouquin and R. Li²⁹. The His and p97 antibodies were purchased from Qiagen and Research Diagnostics, respectively.

Pulse-chase labelling and cycloheximide chase analysis

Pulse-chase experiments and immunoprecipitations were performed essentially as described¹⁷. Cells were grown at 30 °C to mid-log phase, and were labelled for 10 min at the same temperature or shifted to non-permissive temperature for 20 min before labelling. For cycloheximide chase experiments, cells with an absorbance of 10.0 at 600 nm were resuspended in 2.5 ml YPD medium containing 100 μg ml⁻¹ cycloheximide. Samples were taken at the different time points and proteins were prepared for immunoblotting as described¹².

Cellular fractionation and protease protection experiments

Cells with an absorbance of 25 at 600 nm were taken at the indicated time points during cycloheximide chase. Spheroplasts were made and homogenized as described³⁰. Aliquots of cell lysates were centrifuged for 10 min at 20,000g in a microcentrifuge, and the resulting pellet and supernatant fractions were analysed by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Parallel aliquots were treated with 0.5 mg ml⁻¹ proteinase K on ice for 30 min either in the absence or presence of 1% Triton X-100. We added 20 mM phenylmethylsulphonyl fluoride (PMSF) to inhibit proteinase K before SDS–PAGE analysis.

Purification and characterization of p97

The p97 mutant was generated by mutating codons E305 and E578 to Q in plasmid pQE-p97 (ref. 19) using the Stratagene Quickchange method. The sequence was confirmed. Rat liver p97 and recombinant p97 were purified as described^{5,19}. Binding experiments of p97 to glutathione S-transferase fusion proteins were carried out as before¹⁹. We measured the ATPase activity of p97 as described²².

Experiments with mammalian cells

The experiments were essentially performed as described²⁰. Briefly, astrocytoma cells

expressing US11 were incubated for 5 min in the presence of 50 μM proteasome inhibitor ZL₃VS and 27.5 μCi [³⁵S]methionine per 1.0×10^6 cells. After washing, the cells were permeabilized by addition of 0.028% digitonin on ice in the presence of an ATP regenerating system, and unless otherwise indicated, 6 μg p97 or p47 was added for 30 min on ice. The samples were then incubated at 37 °C for different time periods, and separated into membrane and supernatant fractions. Immunoprecipitation with heavy chain antibodies and re-precipitation with ubiquitin antibodies were done as described²⁰. When ubiquitinated chains were analysed, deubiquitination was inhibited by addition of 20 μM ubiquitin and 1 μM ubiquitin aldehyde during the chase. With the exception of the experiment in Fig. 3d, 2 mM N-ethylmaleimide (NEM) was added during sample preparation. To deplete ATP, the ATP regenerating system was omitted and 0.2 U μL^{-1} hexokinase and 50 mM glucose were added. To analyse p97-associated MHC heavy chains, cell fractions were solubilized in 1% deoxyBig CHAP, 30 mM Tris/HCl pH 7.4, 150 mM potassium acetate, 4 mM magnesium acetate, 1 mM ATP and protease inhibitors. Immunoprecipitation was carried out with His antibodies followed by a second pre-precipitation with heavy chain or HA antibodies. For protease protection experiments, 0.35 mg ml⁻¹ proteinase K was added to the fractions and the incubation was carried out for 15 min on ice before addition of 10 mM PMSF.

Received 14 June; accepted 22 October 2001.

1. Bonifacino, J. S. & Weissman, A. M. Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annu. Rev. Cell Dev. Biol.* **14**, 19–57 (1998).
2. Wiertz, E. J. et al. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* **384**, 432–438 (1996).
3. Kihara, A., Akiyama, Y. & Ito, K. Dislocation of membrane proteins in FtsH-mediated proteolysis. *EMBO J.* **18**, 2970–2981 (1999).
4. Leonhard, K. et al. Membrane protein degradation by AAA proteases in mitochondria: extraction of substrates from either membrane surface. *Mol. Cell* **5**, 629–638 (2000).
5. Kondo, H. et al. p47 is a cofactor for p97-mediated membrane fusion. *Nature* **388**, 75–78 (1997).
6. Latterich, M., Fröhlich, K. U. & Schekman, R. Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes. *Cell* **82**, 885–893 (1995).
7. Rabouille, C., Levine, T. P., Peters, J. M. & Warren, G. An NSF-like ATPase, p97 and NSF mediate cisternal regrowth from mitotic Golgi fragments. *Cell* **82**, 905–914 (1995).
8. Acharya, U. et al. The formation of Golgi stacks from vesiculated Golgi membranes requires two distinct fusion events. *Cell* **82**, 895–904 (1995).
9. Ghislain, M., Dohmen, R., Levy, F. & Varshavsky, A. Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin mediated proteolysis in *Saccharomyces cerevisiae*. *EMBO J.* **15**, 4884–4899 (1996).
10. Koegl, M. et al. A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* **96**, 635–644 (1999).
11. Hoppe, T. et al. Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell* **102**, 577–586 (2000).
12. Johnson, E. S., Ma, P. C., Ota, I. M. & Varshavsky, A. A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem.* **270**, 17442–17456 (1995).
13. DeHoratius, C. & Silver, P. A. Nuclear transport defects and nuclear envelope alterations are associated with mutation of the *Saccharomyces cerevisiae* NPL4 gene. *Mol. Biol. Cell* **7**, 1835–1855 (1996).
14. Hitchcock, A. L. et al. The conserved Npl4 protein complex mediates proteasome-dependent membrane-bound transcription factor activation. *Mol. Biol. Cell* **12**, 3226–3241 (2001).
15. Dai, R., Chen, E., Longo, D. L., Gorbea, C. M. & Li, C. C. Involvement of valosin-containing protein, an ATPase co-purified with Ikb α and 26 S proteasome, in ubiquitin-proteasome-mediated degradation of Ikb α . *J. Biol. Chem.* **273**, 3562–3573 (1998).
16. Verma, R. et al. Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. *Mol. Biol. Cell* **11**, 3425–3439 (2000).
17. Casagrande, R. et al. Degradation of proteins from the ER of *S. cerevisiae* requires an intact unfolded protein response pathway. *Mol. Cell* **5**, 729–735 (2000).
18. Hiller, M. M., Finger, A., Schweiger, M. & Wolf, D. H. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science* **273**, 1725–1728 (1996).
19. Meyer, H. H., Shorter, J. G., Seemann, J., Pappin, D. & Warren, G. A complex of mammalian Ufd1 and Npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *EMBO J.* **19**, 2181–2192 (2000).
20. Shamu, C. E., Story, C. M., Rapoport, T. A. & Ploegh, H. L. The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. *J. Cell Biol.* **147**, 45–57 (1999).
21. Lamb, J. R., Fu, V., Wirtz, E. & Bangs, J. D. Functional analysis of the trypanosomal AAA protein TbVCP with *Trans*-dominant ATP hydrolysis mutants. *J. Biol. Chem.* **276**, 21512–21520 (2001).
22. Meyer, H. H., Kondo, H. & Warren, G. The p47 co-factor regulates the ATPase activity of the membrane fusion protein, p97. *FEBS Lett.* **437**, 255–257 (1998).
23. Shamu, C. E., Flierman, D., Ploegh, H. L., Rapoport, T. A. & Chau, V. Polyubiquitination is required for US11-dependent movement of MHC class I heavy chain from the ER into the cytosol. *Mol. Biol. Cell* **12**, 2546–2555 (2001).
24. Dai, R. & Li, C. C. Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. *Nature Cell Biol.* **3**, 740–744 (2001).
25. Zhang, X. et al. Structure of the AAA ATPase p97. *Mol. Cell* **6**, 1473–1484 (2000).
26. Singleton, M. R., Sawaya, M. R., Ellenberger, T. & Wigley, D. B. Crystal structure of T7 gene 4 ring helicase indicates a mechanism for sequential hydrolysis of nucleotides. *Cell* **101**, 589–600 (2000).
27. Schmidt, M., Lupas, A. N. & Finley, D. Structure and mechanism of ATP-dependent proteases. *Curr. Opin. Chem. Biol.* **3**, 584–591 (1999).
28. Matlack, K. E., Misselwitz, B., Plath, K. & Rapoport, T. A. BiP acts as a molecular ratchet during posttranslational transport of Prepro- α factor across the ER membrane. *Cell* **97**, 553–564 (1999).
29. Thuret, J., Valay, J., Faye, G. & Mann, C. C. Cdk1 (CAK *in vivo*), a novel Cdk-activating kinase. *Cell* **86**, 565–576 (1996).
30. Deshaies, R. J. & Schekman, R. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* **105**, 633–645 (1987).

Supplementary Information accompanies the paper on Nature's website (<http://www.nature.com>).

Acknowledgements

We thank C. Shamu, R. Casagrande, A. Hitchcock and N. Bouquin for discussions; D. Schoffnegger for experimental help; C. Shamu, W. Prinz, B. Tsai, D. Flierman, B. DeDecker and D. Finley for critical reading of the manuscript; and G. Warren for support and comments. Y.Y. is supported by the Helen Hay Whitney postdoctoral fellowship. H.M. was supported by the National Institutes of Health (NIH) and Human Frontier Science Program grants and T.A.R. by an NIH grant. T.A.R. is a Howard Hughes Medical Institute Investigator.

Correspondence and requests for material should be addressed to T.A.R. (e-mail: tom_rapoport@hms.harvard.edu).

phot1 and phot2 mediate blue light regulation of stomatal opening

Toshinori Kinoshita^{*†}, Michio Doi^{*†}, Noriyuki Suetsugu^{‡§}, Takatoshi Kagawa^{‡||}, Masamitsu Wada^{‡§} & Ken-ichiro Shimazaki^{*}

^{*} Department of Biology, Faculty of Science, Kyushu University, Ropponmatsu, Fukuoka 810-8560, Japan

[‡] Division of Biological Regulation and Photobiology, National Institute for Basic Biology, Okazaki 444-8585, Japan

[§] Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, Tokyo 192-0397, Japan

^{||} Unit Process and Combined Circuit, PRESTO, Japan Science and Technology Corporation, 1-8, Honcho 4-chome, Kawaguchi, Saitama 332-0012, Japan

[†] These authors contributed equally to this work.

The stomatal pores of higher plants allow for gaseous exchange into and out of leaves. Situated in the epidermis, they are surrounded by a pair of guard cells which control their opening in response to many environmental stimuli, including blue light^{1,2}. Opening of the pores is mediated by K⁺ accumulation in guard cells through a K⁺ channel and driven by an inside-negative electrical potential³. Blue light causes phosphorylation and activation of the plasma membrane H⁺-ATPase that creates this potential^{1,2,4–6}. Thus far, no blue light receptor mediating stomatal opening has been identified⁷, although the carotenoid, zeaxanthin, has been proposed^{2,8}. *Arabidopsis* mutants deficient in specific blue-light-mediated responses have identified^{7,9–14} four blue light receptors, cryptochrome 1 (cry1), cryptochrome 2 (cry2), phot1 and phot2. Here we show that in a double mutant of phot1 and phot2 stomata do not respond to blue light although single mutants are phenotypically normal. These results demonstrate that phot1 and phot2 act redundantly as blue light receptors mediating stomatal opening.

Blue light responses of higher plants include de-etiolation, phototropism, chloroplast relocation, and stomatal opening⁷. cry1 and cry2 mediate de-etiolation⁹, and phot1 and phot2 (nph1 and npl1, respectively¹⁰) mediate phototropism^{11,12} and chloroplast relocation^{12–14}. These blue-light-mediated responses allow plants to capture light energy efficiently, or to avoid damage from high light intensity, thereby optimizing photosynthesis and growth under various light conditions. Photosynthesis is regulated by stomatal aperture.

We considered the phot1 protein and its homologue phot2 as candidates for stomatal blue-light receptors because of their structural properties. The phot1 protein is a serine/threonine protein kinase¹¹ and mediates the increase in cytosolic Ca²⁺ in response to blue light¹⁵. A serine/threonine protein kinase⁶ and an increase in cytosolic Ca²⁺ (refs 2, 16) have both been reported to be involved in

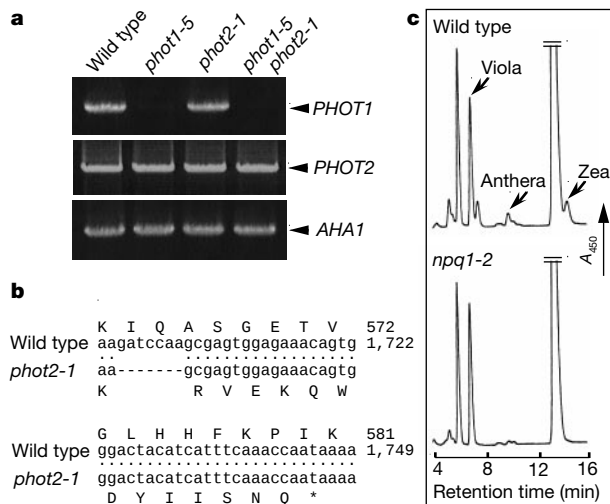


Figure 1 Analyses of *Arabidopsis* blue-light photoreceptor mutants. **a**, RT-PCR analysis using specific primers for *PHOT1*, *PHOT2* and *AHA1*. Complementary DNAs were synthesized from mRNA extracted from leaves. **b**, Partial nucleotide and deduced amino-acid sequences of *PHOT2* cDNAs from the wild type and the *phot2-1* single mutant. Dashes indicate the 7-bp deletion in *phot2-1*. Asterisk shows the predicted in-frame termination codon in *phot2-1*. The amino-acid and nucleotide positions are indicated on the right. **c**, Analysis of zeaxanthin accumulation in leaves of the wild type and *npq1-2* mutant by HPLC in response to red light at $600 \mu\text{mol m}^{-2} \text{s}^{-1}$. A_{450} , absorbance at 450 nm; viola, violaxanthin; anthera, antheraxanthin; zea, zeaxanthin.

blue-light-mediated stomatal opening. Moreover, the absorption spectra of the LOV (light, oxygen, or voltage) domains of *phot1* and *phot2* (refs 12, 17) that function as binding sites for the flavin mononucleotide chromophore closely match the action spectrum for stomatal opening^{18,19}. However, in apparent conflict with the above hypothesis, stomata in an *Arabidopsis phot1* mutant are still able to open in response to blue light²⁰. A possible explanation for this observation could be that *phot2* functions redundantly with *phot1* in this stomatal response, because they have overlapping functions in phototropism and chloroplast relocation¹².

To investigate this, we constructed a double mutant using a null allele of *phot1* (*phot1-5*)¹¹ and a chloroplast avoidance mutant *phot2-1* (*cav1-1*)¹³ in the *Arabidopsis* Columbia ecotype. The *phot1-5* mutant does not undergo phototropic bending in low-intensity light¹¹ and the *phot2-1* mutant is unable to conduct chloroplast avoidance responses¹³. An additional mutant, *npq1-2* (non-photochemical quenching), does not accumulate zeaxanthin because it lacks violaxanthin de-epoxidase²¹. We verified the lesions in these mutants at the genetic or biochemical level.

Figure 1a shows a polymerase chain reaction after reverse transcription of RNA (RT-PCR) analysis of *PHOT1* and *PHOT2* transcripts in *Arabidopsis* leaves. The *PHOT1* messenger RNA was expressed in wild-type and *phot2* plants, but was not from *phot1* and double-mutant *phot1 phot2* plants. The *PHOT2* mRNA was found in wild-type and *phot1* plants, and was also found in *phot2* and double-mutant plants. However, *PHOT2* mRNA in *phot2* and double-mutant plants contained a deletion of 7 base pairs (bp) as shown in Fig. 1b. This deletion introduced a stop codon between the

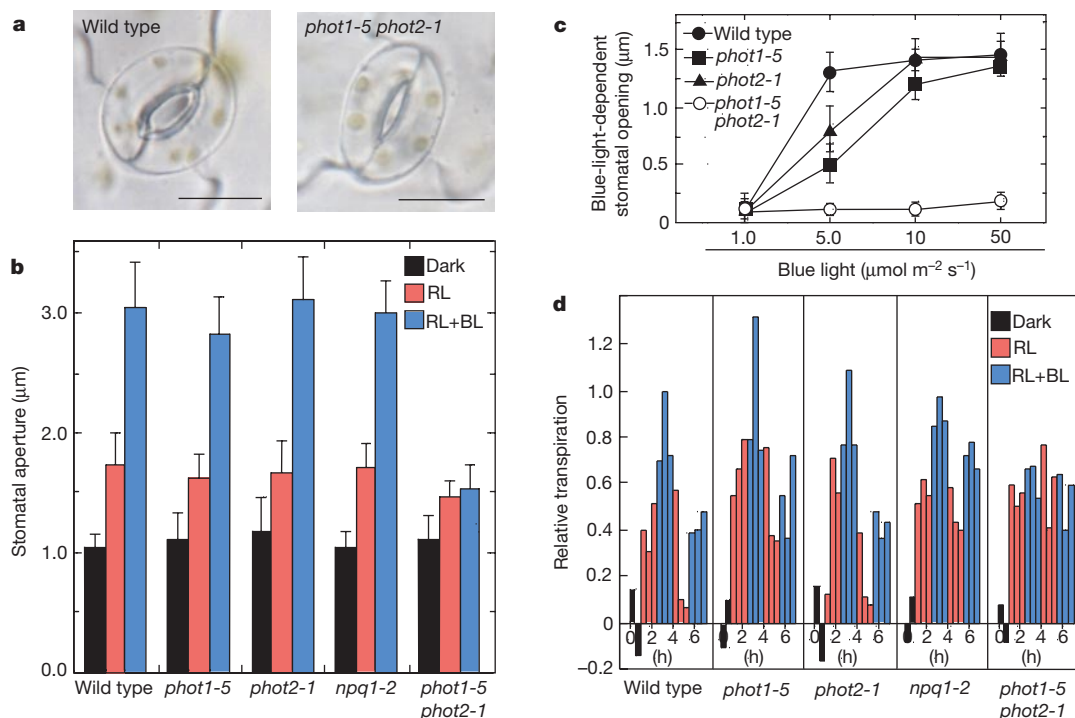


Figure 2 Stomatal opening under blue light. **a**, *Arabidopsis* stomata in the wild type (left) and *phot1-5 phot2-1* double-mutant (right) plants. Epidermal strips were illuminated with blue light under background red light for 2 h. The scale bar represents 10 μm . **b**, Stomatal apertures under different light conditions in a wild type, single mutants of *phot1-5*, *phot2-1*, *npq1-2* and the *phot1-5 phot2-1* double mutant. Stomatal opening was induced by red light (RL), and red and blue light (RL + BL) as in **a**. Each bar indicates a mean of 45 measurements with standard deviations. **c**, Fluence rate dependency of stomatal opening in response to blue light. The ordinate axis shows net blue-light-dependent stomatal opening. The opening measurements were obtained by subtraction of the aperture under red light from that under red and blue light. Stomatal opening was

induced as in **a**. Each point indicates a mean of 45 measurements with standard deviations. **d**, Time courses of the change in transpiration in whole *Arabidopsis* plants in response to red and blue light. The seedlings in the test tube were kept in the dark for 1 h, then illuminated with red light for 1.5 h, and then superimposed with blue light for 1.5 h. After that, the seedlings were returned to red light for 1.5 h upon turning off the blue light, followed by the second superimposition with blue light for 1.5 h. The weight of a test tube containing the seedlings were measured every 0.5 h, and the decrease in the weight was regarded as the water loss by transpiration. Light-dependent transpiration was calculated by subtraction of transpiration and evaporation in the dark. Each bar represents relative transpiration for each 0.5-h period.

LOV2 and kinase domain, so active phot2 protein was not produced in the mutant plants. The *AHA1* (*Arabidopsis* plasma membrane H^+ -ATPase 1) mRNA was found in all these plants. Red-light-induced accumulation of zeaxanthin was also analysed in *npq1-2* mutants using high-performance liquid chromatography (HPLC). Under these conditions, zeaxanthin accumulated in wild-type, but not in *npq1-2* plants (Fig. 1c). The *phot1 phot2* double mutant also accumulated zeaxanthin (data not shown). Leaf periphery of the double mutant curled downward (abaxial side), and the widths of the leaves appeared to be narrower in the double mutant than in the wild type and single mutants. Growth rate was slightly reduced in the double mutant under our growth condition.

Next, epidermal strips from wild-type plants, single mutants of *phot1-5*, *phot2-1*, *npq1-2*, and a double mutant *phot1-5 phot2-1*, were used to investigate stomatal responses. The stomata closed in the dark but opened slightly under red light illumination of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ in strips from all the plants tested (Fig. 2b). The stomata opened much wider when a low-fluence-rate blue light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) was added to the background of red light in wild-type tissue, and in tissues from single mutants of *phot1-5*, *phot2-1*

and *npq1-2*. However, the stomata did not open in the *phot1-5 phot2-1* double mutant (Fig. 2a, b), which suggests that *phot1* and *phot2* are, in fact, the photoreceptors for stomatal opening. The responses were blue-light-specific because the same intensity of red light alone ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) had no significant effect. The slight opening observed under red light might be mediated by photosynthesis²². The blue-light-specific stimulation of stomatal opening in the *npq1-2* mutant is in good agreement with a recent result obtained using gas exchange measurements²³.

We then investigated the dependency of stomatal opening on blue-light fluence, and found different sensitivities for the wild type and the single mutants of *phot1-5* and *phot2-1* (Fig. 2c). When blue light of $1 \mu\text{mol m}^{-2} \text{s}^{-1}$ was added to the background red light, no effect of the blue light was observed in any of the plants tested. When the blue-light fluence rate was increased to $5 \mu\text{mol m}^{-2} \text{s}^{-1}$, the stomata opened fully in the wild-type tissues, but opened only partially in both of the single mutants. In most cases, the stomata in *phot2-1* opened wider than in *phot1-5* under these conditions. No difference in stomatal opening was observed among all plants tested, except in the double mutant under $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ or higher fluence rates (Fig. 2c). No blue-light-dependent response was observed in the double mutant. These results indicate that the single mutation in the *PHOT1* and *PHOT2* genes decreased the sensitivity to blue light. Taken together, these results suggest that both *phot1* and *phot2* work simultaneously and redundantly as blue-light receptors, at least under low-light intensity conditions (around $5 \mu\text{mol m}^{-2} \text{s}^{-1}$ or less).

However, it is possible that the double mutation affected the activity of the plasma membrane H^+ -ATPase, which drives stomatal opening, and results in the prevention of stomatal opening. We are able to rule this possibility out because the amount of the plasma membrane H^+ -ATPase did not differ in these plants when determined by immunological methods (Fig. 3a). Moreover, the stomata opened widely in the double mutant as well as in other plants when the guard-cell plasma membrane H^+ -ATPase was directly activated using the fungal toxin fusicoccin²⁴ (Fig. 3b). This result suggests that other downstream components, including voltage-gated inward-rectifying K^+ channels³ and metabolic pathways²⁵ that are involved in stomatal opening, are not affected by the photoreceptor mutations. We therefore conclude that the simultaneous impairment of *PHOT1* and *PHOT2* genes resulted in loss of blue-light-mediated stomatal opening, probably through a lack of blue-light perception.

Essentially the same results as above were obtained using the intact *Arabidopsis* plants. When whole plants were illuminated with red light at $45 \mu\text{mol m}^{-2} \text{s}^{-1}$, transpiration increased in all the plants tested (Fig. 2d). However, this stomatal opening might be mediated by photosynthesis. When a low fluence rate of blue light at $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ was superimposed on the background red light, transpiration was stimulated by twofold in wild-type plants, as well as single mutants of *phot1-5*, *phot2-1* and *npq1-2*. Transpiration was not stimulated by blue light in the double mutant *phot1-5 phot2-1*. When a low fluence rate of red light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) instead of blue light was superimposed on the background red light, transpiration was not stimulated significantly (data not shown). This effect of blue light on stomatal opening was observed reproducibly (Fig. 2d). Upon removal of blue light, transpiration decreased

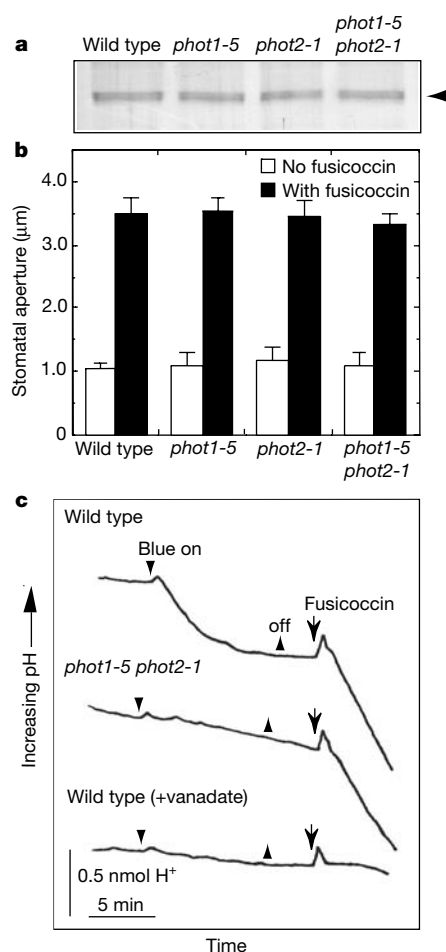


Figure 3 Amount of plasma membrane H^+ -ATPase, fusicoccin-induced stomatal opening, and blue light-dependent H^+ extrusion in *Arabidopsis* blue light photoreceptor mutants. **a**, Western blot analysis of plasma membrane H^+ -ATPase. Twenty micrograms of protein extracted from rosette leaves was loaded on each lane. **b**, Stomatal opening induced by fusicoccin in the dark. Stomatal apertures were determined 2 h after the addition of $10 \mu\text{M}$ fusicoccin. Means of 45 measurements are presented with standard deviations. **c**, H^+ extrusion in the epidermal strips in response to blue light. Intensities of blue light and red light were 10 and $50 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. Fusicoccin was added at $10 \mu\text{M}$. Vanadate was added at 0.5 mM . A typical result of three independent experiments is presented.

Table 1 Blue-light-dependent H^+ extrusion in *Arabidopsis* epidermal strips

	Maximum rate of H^+ extrusion ($\text{nmol } H^+ \text{ h}^{-1} \text{ cm}^{-2}$)	
	Blue light	Fusicoccin
Wild type	8.95 ± 0.85	11.92 ± 0.51
<i>phot1-5</i>	7.78 ± 1.18	10.68 ± 0.80
<i>phot2-1</i>	8.26 ± 2.15	10.63 ± 1.19
<i>npq1-2</i>	6.59 ± 2.56	9.01 ± 0.93
<i>phot1-5 phot2-1</i>	0	11.33 ± 1.67

Means of three determinations are presented with standard deviations. Other experimental conditions were the same as in Fig. 3c.

gradually, probably owing to the sustained effect of blue light on stomatal opening^{4,5,26}, but increased again if blue light was reapplied. However, blue light did not increase transpiration in *phot1 phot2* double mutants. Taking together the results obtained using both epidermal strips and intact plants indicates that *phot1* and *phot2* act as blue-light receptors mediating stomatal opening, and function redundantly.

Stomatal opening is mediated by a plasma membrane H⁺-ATPase, and epidermal strips extrude H⁺ in response to blue light via this enzyme⁴⁻⁶. We would therefore predict that epidermal strips taken from an *Arabidopsis phot1 phot2* double mutant would be unable to respond to blue light in this manner. As expected, the epidermal strips from wild-type plants indeed actively extruded H⁺ in response to blue light, but the strips from the double mutant did not (Fig. 3c). However, fusicoccin, an activator of H⁺-ATPase, elicited H⁺ extrusion in both plants, indicating that the lack of the H⁺ extrusion was not due to a lesion in the H⁺-ATPase in the double mutant, but to lack of blue-light signal perception. The H⁺ extrusions in response to blue light were similarly observed in single mutants of *phot1*, *phot2* and *npq1* (Table 1). These H⁺ extrusions were inhibited by vanadate, an inhibitor of the H⁺-ATPase.

The *phot1* and *phot2* proteins are not only the photoreceptors for stomatal opening, but also the photoreceptors for phototropism and chloroplast relocation movement. All three of these physiological responses serve to improve the efficiency of photosynthesis. Stomatal opening provides CO₂ for photosynthesis and stimulates production of NADP and ADP in the Calvin cycle, which not only increases the efficiency of light-energy capture, but also prevents the generation of toxic active-oxygen species. Phototropic bending of stems serves to orient leaf surfaces perpendicular to incident light, maximizing the light-energy capture by photosynthetic organs⁷. Chloroplasts accumulate under weak light at the cell surface perpendicular to the incident light to maximize light capture, but under strong light move to the cell wall parallel to the light to minimize damage¹²⁻¹⁴. The *phot1* and/or *phot2* photoreceptors mediate all of these responses. As the *phot* family of proteins is found only in plants and functions in regulation of photosynthesis efficiency, the emergence of *phot* proteins must have played an important role in plant evolution, increasing the plant's chances for success. Stomatal opening, phototropic bending and chloroplast relocation are very different events so it is interesting to discover that the same photoreceptor can mediate a diverse range of responses in plant cells. □

Methods

Plant materials

The Columbia ecotype *Arabidopsis* was used for all the studies reported here. Wild-type plants, the mutants of *phot1-5* (*nph1-5*)¹¹, *phot2-1* (*npl1-1* or *cav1-1*)¹³ and *npq1-2* (ref. 21) and a double mutant of *phot1-5 phot2-1* (*nph1-5 npl1-1*) were grown with 16 h of light (6:00 to 22:00)/8 h of dark under fluorescent lamp (50 μmol m⁻² s⁻¹) on soil irrigated with mineral nutrients. The temperature was maintained at 22 °C, and the relative humidity at 50%. Wild-type and *npq1-2* mutant plants were obtained from the *Arabidopsis* Biological Resource Center (ABRC).

RT-PCR analysis

RNA was extracted from green leaves of 5-week-old *Arabidopsis* seedlings²⁷. First-strand complementary DNA was synthesized from this total RNA using oligo(dT)₁₂₋₁₈ as a primer (Takara). Three genes for the *PHOT1* (GenBank accession number AF030864), *PHOT2* (AF053941), and *AHA1* (M24107) were amplified from the first-strand cDNA by PCR with specific primers. The primers we used were: 5'-CAGAGTGGGATTGGTTTGAAG-3' and 5'-GTTTGTCTATCTTAGCTCAGG-3' for the *PHOT1*; 5'-CGAGTGGAAAAGAGACACATGG-3' and 5'-CTTGTCCGTAGAATTCACAAGCAC-3' for the *PHOT2*; and 5'-TTCTTCTGGGTGAAGATGTCAGG-3' and 5'-GTTTGTGTTGTTGTGCAACTCCAAC-3' for the *AHA1*. The amplified DNA fragments covering 416–3,041 of *PHOT1*, 93–2,736 of *PHOT2*, and 79–2,966 bp of *AHA1* were confirmed by sequence analysis (ABI PRISM 3100, PE Applied Biosystems).

Stomatal aperture

Stomatal apertures were determined using 'mature stomata' in epidermal strips from 3- to 5-week-old plants of *Arabidopsis thaliana* L. (cv. Columbia)²⁸. Leaves were collected in the

early morning. The adaxial side of leaves was attached to a piece of adhesive tape and epidermal strips were peeled off from the abaxial side of the leaf under dim light using forceps. The strips were floated on 3 ml of basal reaction mixture in Petri dishes (20 mm diameter) and kept in the dark for 1 h. Strips were then illuminated with red light, or with blue light superimposed on the background red light, for 2 h. Basal reaction mixture contained 5 mM Mesbistrispropane (BTP) pH 6.5, 50 mM KCl, and 0.1 mM CaCl₂. Stomatal apertures were measured from transmission images obtained using a laser scanning microscope (Carl Zeiss, LSM-410) at 900-fold magnification. The laser wavelength used was 568 nm. All measurements were made between 8:00 and 14:00, at a temperature of 24 °C.

Transpiration

Transpiration was determined every 0.5 h by the decrease in the weight of a glass tube in which *Arabidopsis* seedlings were placed according to a modification of the method of ref. 29. The roots of two intact 3- to 5-week-old *Arabidopsis* seedlings were dipped in distilled water inside glass tubes. The seedlings in the tube were kept in the dark for 1 h, then illuminated with red light at 45 μmol m⁻² s⁻¹ for 1.5 h, with added blue light at 10 μmol m⁻² s⁻¹ for 1.5 h. Then the seedlings were returned to red light for 1.5 h upon turning off the blue light, followed by the second illumination with blue light for 1.5 h. Illumination was done from the upper side. All the experiments were performed between 8:00 and 16:00, at a temperature of 24 °C. Light-dependent transpiration was calculated by subtraction of transpiration and evaporation in the dark.

Immunological estimation of H⁺-ATPase

Arabidopsis plasma membrane H⁺-ATPase was detected immunologically using antibodies raised against recombinant VHA1 (*Vicia* H⁺-ATPase)⁶. Leaves were disrupted in the extraction buffer containing 50 mM MOPS-KOH of pH 7.5, 2.5 mM EDTA, 100 mM NaCl, 0.5 mM phenylmethylsulphonyl fluoride, 10 μM leupeptin, and 1 mM dithiothreitol, using a mortar and pestle. Twenty micrograms of protein was loaded and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined by the method of ref. 30.

H⁺ extrusion

Rate of H⁺ extrusion from *Arabidopsis* epidermal strips was determined by measuring the pH decrease in the medium^{5,6}. The medium pH was measured using a pH electrode (Fuji Chemical Measurement, SE-1800GC) connected to a pH meter (Beckman, Model 71). The epidermal strips were treated with ultrasonication for 3 s to remove epidermal and mesophyll cells. The basal reaction mixture (200 μl) contained 0.05 mM Mes-BTP pH 7.0, 50 mM KCl, 0.1 mM CaCl₂, and the epidermal strips of about 0.7 cm². The area of the strips was determined by NIH software in each measurement. Blue light at 10 μmol m⁻² s⁻¹ was provided for 10 min superimposed on background red light at 50 μmol m⁻² s⁻¹. Fusicoccin was added at 10 μM. Vanadate was added at 0.5 mM. All measurements were performed at a temperature of 24 °C.

Light sources

For illumination of epidermal strips, red light was given from a tungsten lamp (Sylvania EXR 150 W) by passing the light through a red glass filter (Corning 2-61). Blue light was given from a tungsten lamp (Sylvania EXR 150 W) through a blue glass filter (Corning 5-60). For the illumination of intact seedlings, red (peak wavelength, 660 nm) and blue light (peak wavelength, 470 nm) were given from light-emission diodes (Model 03300-019, Iwasaki Electric). Fluence rates were determined using a quantum meter (Li-Cor, model 185A).

Received 3 August; accepted 9 October 2001.

- Assmann, S. M. Signal transduction in guard cells. *Annu. Rev. Cell Biol.* **9**, 345–375 (1993).
- Schroeder, J. I., Allen, G. J., Hugouvieux, V., Kwak, J. M. & Waner, D. Guard cell signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 627–658 (2001).
- Schroeder, J. I., Raschke, K. & Neher, E. Voltage dependence of K⁺ channels in guard cell protoplasts. *Proc. Natl Acad. Sci. USA* **84**, 4108–4112 (1987).
- Assmann, S. M., Simoncini, L. & Schroeder, J. I. Blue light activates electrogenic ion pumping in guard cell protoplasts of *Vicia faba* L. *Nature* **318**, 285–287 (1985).
- Shimazaki, K., Iino, M. & Zeiger, E. Blue light-dependent proton extrusion by guard-cell protoplasts of *Vicia faba*. *Nature* **319**, 324–326 (1986).
- Kinoshita, T. & Shimazaki, K. Blue light activates the plasma membrane H⁺-ATPase by phosphorylation of the C-terminus in stomatal guard cells. *EMBO J.* **18**, 5548–5558 (1999).
- Briggs, W. R. & Huala, E. Blue-light photoreceptors in higher plants. *Annu. Rev. Cell Dev.* **15**, 33–62 (1999).
- Zeiger, E. & Zhu, J. Role of zeaxanthin in blue light photoreception and modulation of light-CO₂ interactions in guard cells. *J. Exp. Bot.* **49**, 433–442 (1998).
- Ahmad, M. & Cashmore, A. R. HY4 gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature* **366**, 162–166 (1993).
- Briggs, W. R. *et al.* The phototropin family of photoreceptors. *Plant Cell* **13**, 993–997 (2001).
- Huala, E. *et al.* *Arabidopsis* NPH1: A protein kinase with a putative redox-sensing domain. *Science* **278**, 2121–2123 (1997).
- Sakai, T. *et al.* *Arabidopsis* nph1 and npl1: Blue light receptors that mediate both phototropism and chloroplast relocation. *Proc. Natl Acad. Sci. USA* **98**, 6969–6974 (2001).
- Kagawa, T. *et al.* *Arabidopsis* NPL1: A phototropin homolog controlling the chloroplast high-light avoidance response. *Science* **291**, 2138–2141 (2001).
- Jarillo, J. A. *et al.* Phototropin-related NPL1 controls chloroplast relocation induced by blue light. *Nature* **410**, 952–954 (2001).

15. Baum, G., Long, J. C., Jenkins, G. I. & Trewavas, A. J. Stimulation of the blue light phototropic receptor NPH1 causes a transient increase in cytosolic Ca^{2+} . *Proc. Natl Acad. Sci. USA* **96**, 13554–13559 (1999).
16. Shimazaki, K., Goh, C. H. & Kinoshita, T. Involvement of intracellular Ca^{2+} in blue light-dependent proton pumping in guard cell protoplasts from *Vicia faba*. *Physiol. Plant.* **105**, 554–561 (1999).
17. Christie, J. M., Salomon, M., Nozue, K., Wada, M. & Briggs, W. R. LOV (light, oxygen, or voltage) domains of the blue light photoreceptor phototropin (nph1): Binding sites for the chromophore flavin mononucleotide. *Proc. Natl Acad. Sci. USA* **96**, 8779–8783 (1999).
18. Karlsson, P. E. Blue light regulation of stomata in wheat seedlings. II. Action spectrum and search for action dichroism. *Physiol. Plant.* **66**, 207–210 (1986).
19. Eisinger, W., Swartz, T. E., Bogomoloi, R. A. & Taiz, L. The ultraviolet action spectrum for stomatal opening in broad bean. *Plant Physiol.* **122**, 99–105 (2000).
20. Lasceve, G. et al. *Arabidopsis* contains at least four independent blue-light-activated signal transduction pathways. *Plant Physiol.* **120**, 605–614 (1999).
21. Niyogi, K. K., Grossman, A. R. & Bjorkman, O. *Arabidopsis* mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. *Plant Cell* **10**, 1121–1134 (1998).
22. Schwartz, A. & Zeiger, E. Metabolic energy for stomatal opening. Role of photophosphorylation and oxidative phosphorylation. *Planta* **161**, 129–136 (1984).
23. Eckert, M. & Kaldenhoff, R. Light-induced stomatal movement of selected *Arabidopsis thaliana* mutants. *J. Exp. Bot.* **51**, 1435–1442 (2000).
24. Ballio, A. et al. Fusicoccin: A new wilting toxin produced by *Fusicoccum amygdali* Del. *Nature* **203**, 297 (1964).
25. Willmer, C. & Fricker, M. D. *Stomata* 2nd edn (Chapman & Hall, London, 1996).
26. Iino, M., Ogawa, T. & Zeiger, E. Kinetic properties of blue light response of stomata. *Proc. Natl Acad. Sci. USA* **82**, 8019–8023 (1985).
27. Chomczynski, P. & Sacchi, N. Single step-method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159 (1987).
28. Merlot, S., Gosti, F., Guerrier, D., Vasseure, A. & Giraudat, J. The ABI1 and ABI2 protein phosphatase 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. *Plant J.* **25**, 295–303 (2001).
29. Kondo, N. & Sugahara, K. Changes in transpiration rate of SO_2 -resistant and -sensitive plants with SO_2 fumigation and the participation of abscisic acid. *Plant Cell Physiol.* **19**, 365–373 (1978).
30. Bradford, M. M. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254 (1976).

Acknowledgements

We thank J. Silverthorne for a critical reading of the manuscript. This work was supported in part by a grant from the research fellowships of the Japan Society for the Promotion of Science for Young Scientists to T. Kinoshita and N.S., a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan to K.S. and M.W., and Kyushu University Interdisciplinary Programmes in Education and Projects in Research Development to K.S. This work was also supported partly by the PROBRAIN and NOVARTIS to M.W. and partly by a grant from PRESTO, Japan Science and Technology Corporation (T. Kagawa).

Correspondence and requests for materials should be addressed to K.S.
(e-mail: kenrcb@mbbox.nc.kyushu-u.ac.jp).

AID is required to initiate Nbs1/ γ -H2AX focus formation and mutations at sites of class switching

Simone Petersen*, Rafael Casellas‡, Bernardo Reina-San-Martin‡, Hua Tang Chen*, Michael J. Difilippantonio§, Patrick C. Wilson‡, Leif Hanitsch‡, Arkady Celeste*, Masamichi Muramatsu||, Duane R. Pilch¶, Christophe Redon¶, Thomas Ried§, William M. Bonner¶, Tasuku Honjo||, Michel C. Nussenzweig†‡ & André Nussenzweig*†

* Experimental Immunology Branch; § Genetics Branch; and ¶ Laboratory of Molecular Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

‡ Laboratory of Molecular Immunology, The Rockefeller University, and Howard Hughes Medical Institute, New York, New York 10021, USA

|| Department of Medical Chemistry, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan

† These authors contributed equally to this work

Class switch recombination (CSR) is a region-specific DNA recombination reaction that replaces one immunoglobulin heavy-chain constant region (CH) gene with another. This enables a single variable (V) region gene to be used in conjunction with

different downstream CH genes, each having a unique biological activity. The molecular mechanisms that mediate CSR have not been defined, but activation-induced cytidine deaminase (AID), a putative RNA-editing enzyme, is required for this reaction¹. Here we report that the Nijmegen breakage syndrome protein (Nbs1) and phosphorylated H2A histone family member X (γ -H2AX, also known as γ -H2afx), which facilitate DNA double-strand break (DSB) repair^{2–4}, form nuclear foci at the CH region in the G1 phase of the cell cycle in cells undergoing CSR, and that switching is impaired in *H2AX*^{−/−} mice. Localization of Nbs1 and γ -H2AX to the Igh locus during CSR is dependent on AID. In addition, AID is required for induction of switch region (μ)-specific DNA lesions that precede CSR. These results place AID function upstream of the DNA modifications that initiate CSR.

AID, a recently characterized protein belonging to the RNA-editing cytidine deaminase family, is expressed specifically in B lymphocytes undergoing CSR and somatic hypermutation¹. Human and mouse B cells lacking AID appear to develop normally and respond to challenge with antigens; however, they fail to hypermutate their immunoglobulin V genes or undergo CSR^{1,5}. CSR joins immunoglobulin switch regions by looping out and excision of intervening DNA. However, the mechanisms of DNA cleavage, switch region synapsis, and DNA repair after cleavage are not well defined.

To determine whether DNA repair factors associate with DSBs at the switch regions, we first examined the intracellular localization of γ -H2AX, Nbs1, Rad51 and Brca1 in activated B cells by immunofluorescence. Brca1 and Rad51 are required for homologous recombination, the Mre11–Rad50–Nbs1 complex has been implicated in both homologous recombination and non-homologous end-joining (NHEJ), and γ -H2AX is critical for recruiting these repair factors to DSBs⁶ and facilitates NHEJ in *Saccharomyces cerevisiae*⁴. All four proteins showed diffuse nuclear staining in most of the resting B cells from C57BL/6 wild-type mice. High local concentrations of these factors (nuclear foci) were detected in a very small percentage of cells (<5%), which increased significantly when the cells were stimulated to undergo CSR *in vitro* with lipopolysaccharide (LPS) and interleukin (IL)-4 (Fig. 1a). After 3 days of stimulation, 37% of the B cells contained discrete Brca1 foci (12 ± 6 per cell) and 43% contained Rad51 foci (7 ± 3 per cell), consistent with previous results⁷; the remaining cells exhibited a weak, diffuse pattern of nuclear staining (Fig. 1a). Many of the stimulated B cells also formed Nbs1 foci (32% contained, on average, 3 ± 2 per cell) and γ -H2AX foci (40% contained, on average, 4.5 ± 3 per cell). To determine which of these repair factors are co-localized in activated B cells, we performed two colour immunofluorescence experiments (Fig. 1b). Only 20% of the cells that contained Rad51 and Nbs1 ($n = 687$) or Brca1 and Nbs1 foci ($n = 431$) exhibited co-localization. In contrast, γ -H2AX foci co-localized with Nbs1 in 79% of the cells ($n = 354$). Thus, DNA-repair focus formation is induced in B cells by LPS and IL-4, and most of the Nbs1 foci co-localize with γ -H2AX, but less frequently with Brca1 or Rad51 foci.

The Nbs1/ γ -H2AX/Brca1/Rad51 nuclear foci observed in activated B cells may represent repair-complex storage sites, replication-associated DNA damage⁸, telomeric complexes⁹, or a specific response to CSR-induced breaks. To determine whether Nbs1/ γ -H2AX/Brca1/Rad51 foci are associated with sites of CSR, we performed immunocytochemistry staining followed by fluorescence *in situ* hybridization (ICC-FISH) to simultaneously visualize DNA (Igh, TCR α , or immunoglobulin light chain (Ig κ) loci) and protein (Nbs1, γ -H2AX, Brca1 or Rad51) in lymphocytes stimulated with LPS and IL-4 (Fig. 1c)¹⁰. Approximately 15% of cells in a given optical section contained at least one Nbs1 or γ -H2AX focus. Coincidence of either signal with one or both Igh alleles was detected in 69% of the cells with Nbs1 foci and 76% of the cells with γ -H2AX foci (Fig. 1c and Table 1). This co-localization was specific as only 3–5% of the stimulated B cells showed co-localiza-

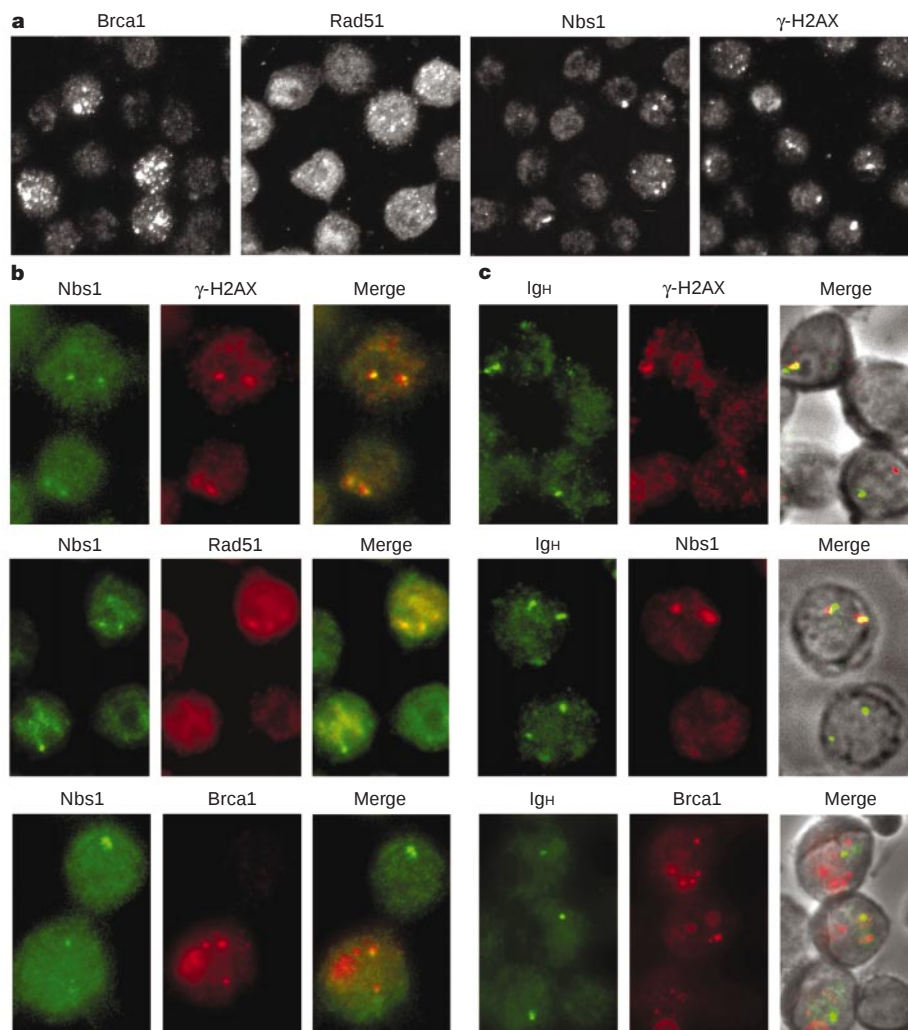


Figure 1 DNA repair foci in wild-type B lymphocytes after stimulation with LPS and IL-4 for 72 h. **a**, Distribution of Brca1, Rad51, Nbs1 and γ -H2AX in activated wild-type B cells. Confocal images were optically sectioned at 0.5- μ m intervals and merged into a maximum projection. **b**, Double staining with Nbs1 (green) together with γ -H2AX (red), Rad51 (red) or Brca1 (red). The images were merged to determine co-localization (yellow). **c**, Co-localization of DNA repair foci with the IgH locus. B cells were stained with anti- γ -H2AX, anti-Nbs1, or anti-Brca1 antibodies (ICC (red)) followed by DNA FISH (green) detection of the CH region. Cells were visualized by phase-contrast microscopy and the images were merged to determine co-localization (yellow). Fluorescence images in **b** and **c** represent a single optical section.

tion of Nbs1 or γ -H2AX with TCR α or Ig κ (Table 1). In contrast, neither Rad51 nor Brca1 foci significantly co-localized with IgH (Table 1), although the average number of these foci per cell was greater than the number of Nbs1 or γ -H2AX foci. DNA DSBs arising from immunoglobulin V gene somatic hypermutation^{11–13} could potentially recruit DNA repair factors, but there was no significant immunoglobulin gene mutation detected in B cells stimulated under these same conditions (two changes in 10,620 V κ nucleotides were found in activated B cells carrying a pre-arranged light chain gene). These findings indicate that Nbs1 and γ -H2AX, but not Brca1 or Rad51, are recruited to DNA lesions created during CSR.

In vertebrates, DNA repair by NHEJ occurs primarily during the G1 phase of the cell cycle, whereas homologous recombination is restricted to S/G2 phases. To determine in which phase of the cell cycle CSR occurs, live B cells were electronically sorted 2 days after stimulation into G1/early S and late S/G₂/M phases, and then examined by ICC-FISH for foci associated with CH (Fig. 2a). Most of the cells (81%) that formed γ -H2AX or Nbs1 foci at the IgH locus were in G1 (Fig. 2a, b). Thus, DNA repair factors are recruited to the CSR sites in the G1 phase of the cell cycle, a result that is consistent with NHEJ as the repair pathway for CSR^{14–16}.

DNA lesions associated with CSR would be expected to form after initiation of germline transcription and before expression of mature switch transcripts^{17,18}. To determine when IgH-associated Nbs1/ γ -H2AX foci appear relative to these events, we assayed germline I γ 1 transcription (I γ 1-C γ 1), mature IgG1 transcripts, S μ -S γ 1 DNA rearrangements, and surface IgG1 expression (Fig. 3a, b). Germline I γ 1 transcription was present 24 h after stimulation. S μ -S γ 1 DNA, mature IgG1 transcripts and surface IgG1 expression

Table 1 Co-localization of protein foci with genomic loci

Genotype	Antibody staining	Cells with foci that co-localize with IgH (%)	Cells with foci that co-localize with TCR α (%)	Cells with foci that co-localize with Ig κ (%)
Wild type	Nbs1	69	3.3	3.7
Wild type	γ -H2AX	76	5.2	3
Wild type	Brca1	8.4	11	5.1
Wild type	Rad51	0	ND	ND
<i>AID</i> ^{-/-}	Nbs1	3.7	2	ND
<i>AID</i> ^{-/-}	γ -H2AX	7	1	ND
<i>AID</i> ^{-/-}	Brca1	5.5	5.9	ND

Co-localization values were measured in B cells after 72 h of LPS and IL-4 culture. For each genotype, all hybridizations were performed in parallel and repeated up to four times. A minimum of 100 cells with foci were examined after each hybridization. ND, not done.

were barely detectable at 48 h, but were clearly present after 72 h. IgH-associated Nbs1 foci began accumulating in B cells 48 h after stimulation (Fig. 3b), after the detection of immunoglobulin germ-line transcripts but before high levels of completed recombination, indicating that focus formation is coincident with initiation of CSR.

To determine the functional significance of γ -H2AX focus formation at sites of CSR, we examined the effects of H2AX ablation on switch recombination. B cells that were isolated from $H2AX^{-/-}$ mice (Supplementary Information Fig. 1) were stimulated with LPS and IL-4 under conditions identical to those used for ICC-FISH analysis. CSR was measured by flow cytometry. B cells from $H2AX^{-/-}$ mice ($n = 8$) exhibited impaired switching as indicated by a 50–86% reduction in surface IgG1 levels relative to littermate controls (Fig. 3c). This deficiency was not due to a gross difference in proliferation as measured by cell number, ^3H -thymidine incorporation ($H2AX^{+/+}$, $2,120 \pm 244$ c.p.m.; $H2AX^{-/-}$, $1,910 \pm 102$ c.p.m. at day 3 of culture), or cell cycle distribution (Fig. 3c).

Furthermore, sterile switch transcripts were induced at similar levels in B cells of $H2AX^{-/-}$ and $H2AX^{+/+}$ mice, but mature IgG1 transcripts and DNA recombination were decreased in the knock-out mice in a manner consistent with the flow cytometric analysis (Fig. 3d). We conclude that H2AX promotes efficient CSR, but it is not essential for the reaction, suggesting that H2AX-independent DNA repair pathways can also process switch DNA lesions.

To determine whether AID is required for switch-region-associated, DNA-repair focus formation, we assayed for co-localization of C_H with Nbs1, γ -H2AX, or Brca1 protein in stimulated B cells from $AID^{-/-}$ mice. In contrast to wild-type B cells, in which a significant fraction of Nbs1/ γ -H2AX foci were detected at the IgH locus, only 4–7% of the cells containing C_H signals and Nbs1 or γ -H2AX foci showed co-localization (Fig. 4c and Table 1). This coincidence was similar to the background frequency observed with the TCR α probe (Table 1). In $AID^{-/-}$ mutant cells, as in wild-type cells, all DNA probes failed to significantly co-localize with Brca1 (Table 1). $AID^{-/-}$ B cells stimulated with LPS and IL-4 were

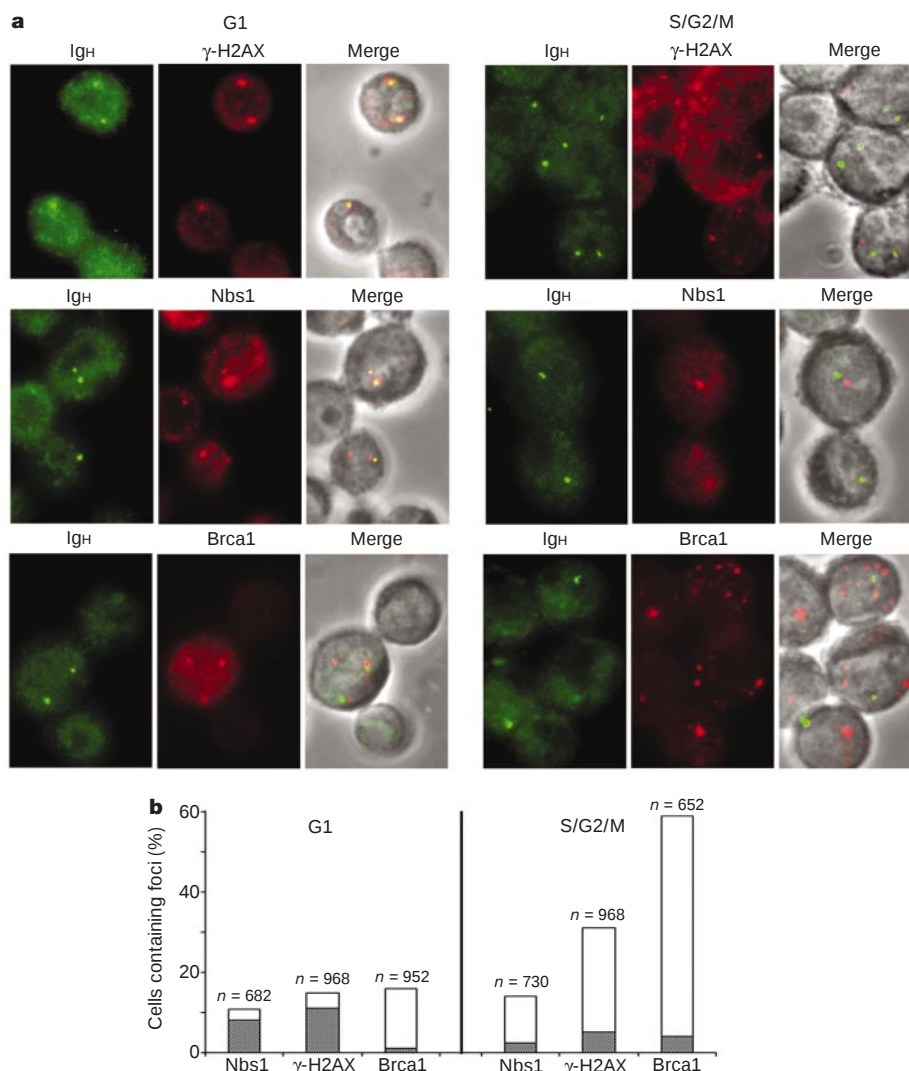


Figure 2 DNA repair foci associated with CSR form predominantly in the G1 phase of the cell cycle. **a**, Representative ICC-FISH images of G1 and S/G2/M sorted cells. Wild-type B cells stimulated for 48 h with LPS and IL-4 were labelled with Hoechst and live cells were electronically separated into G1 (purity >95%) and S/G2/M (purity >80%) cell cycle fractions. Sorted populations were analysed by ICC-FISH for co-localization (yellow) of γ -H2AX, Nbs1 and Brca1 with C_H . **b**, Percentage of sorted cells in a given optical section

that contain Nbs1, γ -H2AX and Brca1 foci (whole bar), and percentage of sorted cells in which DNA repair foci co-localize with C_H (hatched section). Brca1 and γ -H2AX foci were found disproportionately in a greater fraction of S/G2/M cells relative to G1 (γ -H2AX, 31% compared with 15%; Brca1, 59% compared with 16%, respectively), whereas the percentage of G1 cells (11%) and S/G2/M (14%) cells with Nbs1 foci was similar. More than 1,000 cells were analysed.

indistinguishable from those of wild type in terms of the percentage of cells with foci (Nbs1, γ -H2AX, Brca1, or Rad51), the average number of foci per cell and co-localization of these proteins with each other (Fig. 4a, b and data not shown). γ -irradiation of wild-type and *AID*^{-/-} B cells caused a similar increase in the number of foci per cell and the percentage of cells with foci (not shown). This indicates that AID is required specifically for CSR-associated Nbs1/ γ -H2AX focus formation but not for the recruitment of DNA repair factors to DSBs.

To determine whether AID is required to produce DNA lesions in the switch region, we cloned the germline S μ region from *AID*^{-/-} and control B cells that were stimulated with LPS and IL-4. To amplify the unrearranged, highly repetitive and (G+C)-rich S μ region, we used primers that flank germline S μ and Long-Expand Taq polymerase¹⁹. We analysed the first 450 base pairs (bp) of the germline S μ clones, which is a region 5' of the S μ core repeats (starting at nucleotide 4,600; GenBank accession number J00440). We focused on this region because it is frequently involved in CSR²⁰ and might show evidence of aberrant repair of DNA lesions that precede the CSR reaction²¹. Small numbers of mutations, consistent with Long-Expand Taq polymerase error¹⁹, were found in the Igh enhancer (E μ), S μ and the Ig μ constant region (C μ) cloned from resting B cells (Fig. 5a). In contrast, sequence analysis revealed a specific increase in mutation in the S μ region in wild-type B cells stimulated with LPS and IL-4 (Fig. 5a; *P* = 0.0006 for S μ mutation in

B cells stimulated with LPS and IL-4 for 72 h compared with resting B cells (52 mutations/18,660 bp versus 10 mutations/16,542 bp, respectively); *P* = 0.0003 for S μ mutation in stimulated B cells at 72 h compared with E μ plus C μ mutation in B cells stimulated under the same conditions (33 mutations/59,196 bp for E μ plus C μ); *P*-values were determined by a two-tailed *t*-test assuming unequal variance; the frequency of mutation in the 5' region of S μ was 5×10^{-4} when measured with a proofreading polymerase). Consistent with previous reports, we did not find deletions in this part of S μ ²². The induced mutations were AID dependent because no significant increase in mutation over background was observed in S μ in *AID*^{-/-} B cells cultured with LPS and IL-4 (Fig. 5a). These changes in nucleotide sequence were not found on stimulation with CpG (Fig. 5b)—a synthetic oligodeoxynucleotide that induces B-cell proliferation but not CSR. The mutations induced in S μ resembled V gene hypermutation in that they were predominantly single-base changes, and 60% of the changes were in sequence motifs corresponding to hypermutational hot spots²³ (Fig. 5c; *P* = 0.0015, χ^2 test, 31 mutations in 166 hot-spot nucleotides compared with 21 mutations in 284 non-hot-spot nucleotides). Hypermutation has also been reported in *BCL-6* (refs 24, 25) and in areas adjoining switch joints²⁶, but the relationship of these mutations to the switch recombination reaction has never been determined. Our experiments show that the S μ lesions are inducible, that they are S μ specific because they are not found in

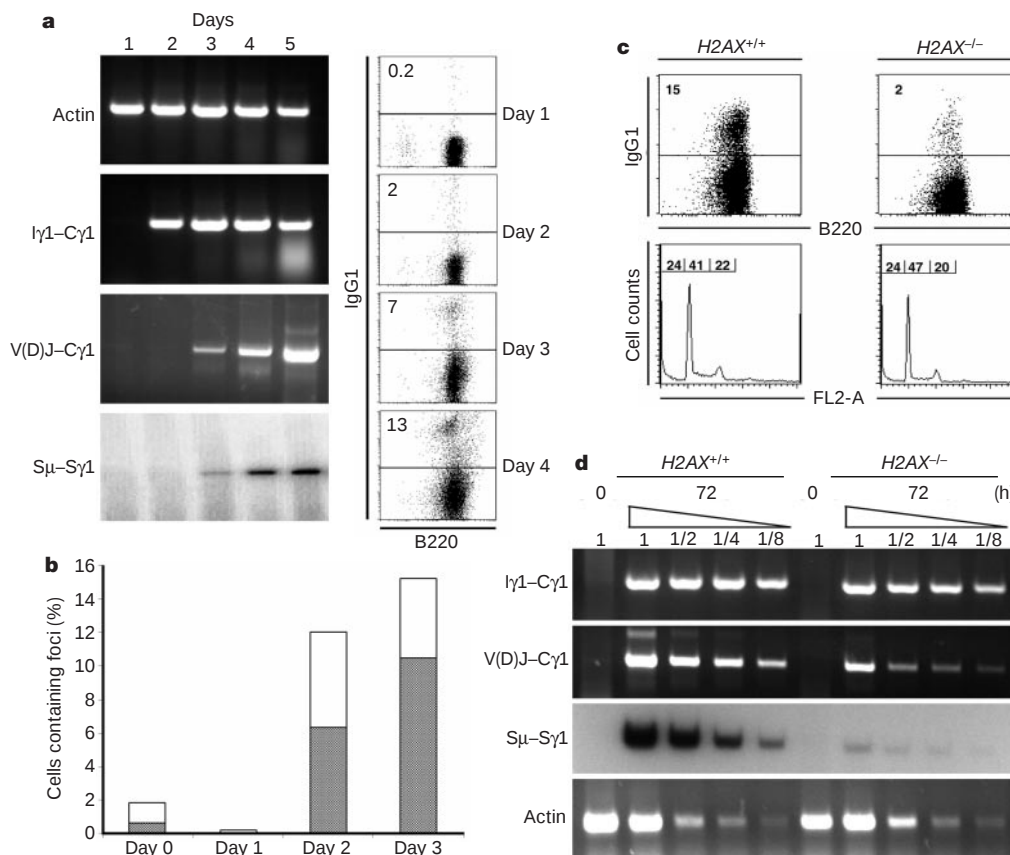


Figure 3 Kinetics of CH-associated foci accumulation in wild-type mice and impaired switching in *H2AX*^{-/-} mice. **a**, Actin control, germline sterile I γ 1-C γ 1 transcripts, mature switch V(D)J-C γ 1 transcripts (assayed by PCR with reverse transcription (RT-PCR)), and S μ -S γ 1 DNA rearrangements assayed by digestion-circularization PCR (DC-PCR) at indicated times of LPS and IL-4 culture. Cell surface expression of IgG1 was detected by flow cytometry. Percentages from total lymphocyte-gated populations are indicated. RT-PCR, DC-PCR and FACS analysis were performed as described¹⁴. **b**, Analysis by ICC-FISH for co-localization of Nbs1 foci with CH, quantified as in Fig. 2b. **c**, Cell surface

expression of IgG1 in B cells from *H2AX*^{-/-} mice and littermates assayed 72 h after stimulation with LPS and IL-4. An aliquot of the samples (shown below) was used to simultaneously measure cell cycle distribution at 72 h. Numbers indicate the percentage of sub-G1, G1 and S/G2/M cells, respectively. **d**, I γ 1-C γ 1 transcripts, mature switch V(D)J-C γ 1 transcripts, and S μ -S γ 1 DNA rearrangements in B cells of *H2AX*^{-/-} and *H2AX*^{+/+} mice assayed at 0 and 72 h after LPS and IL-4 culture. DNA and RNA samples taken at 72 h were diluted as indicated.

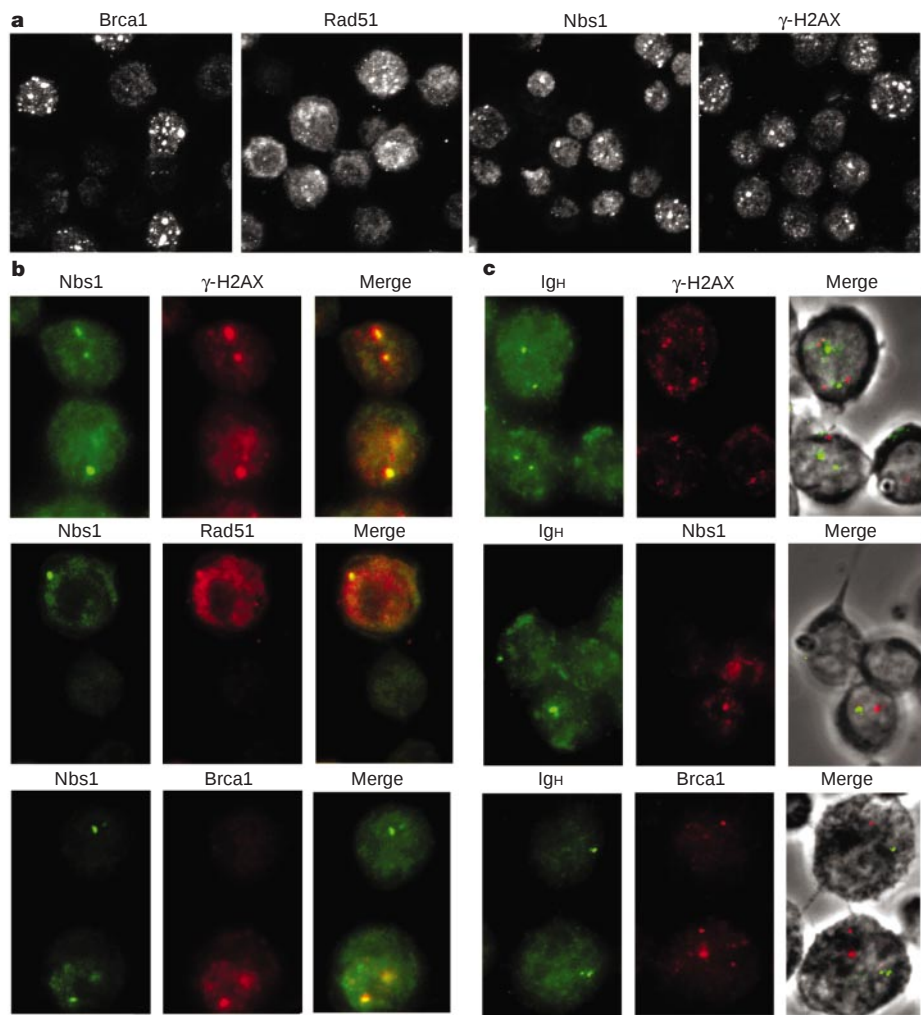


Figure 4 Co-localization of Nbs1 and γ-H2AX at the IgH locus is dependent on AID. **a**, *AID*^{-/-} B cells were stimulated for 72 h with LPS and IL-4 and the intracellular localization of Brca1, Rad51, Nbs1 and γ-H2AX was determined by immunofluorescence as in Fig. 1a. **b**, Double staining with Nbs1 (green) together with either γ-H2AX (red),

Brca1 (red) or Rad51 (red) in *AID*^{-/-} cultures. The images were merged to determine co-localization (yellow). **c**, Co-localization of DNA repair foci with IgH locus in activated *AID*^{-/-} B cells, analysed by ICC-FISH as in Fig. 1c.

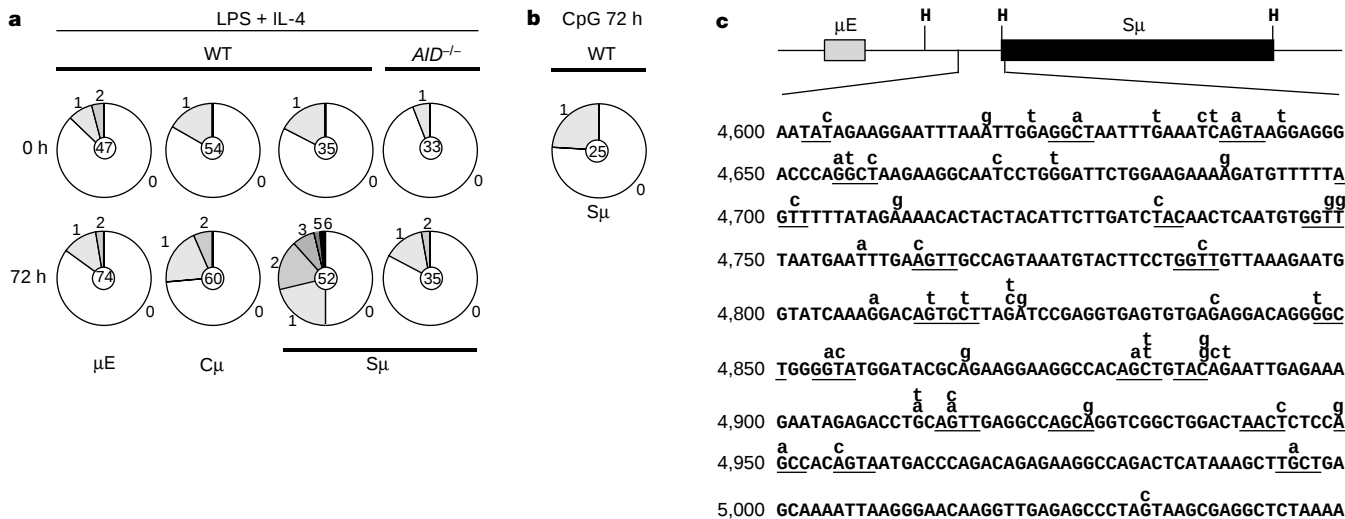


Figure 5 AID-dependent Sμ mutation induced by LPS and IL-4. **a**, Proportion of Eμ, Cμ and Sμ sequences carrying different numbers of mutations before and after stimulation with LPS and IL-4 for 72 h. Segment sizes in the pie charts are proportional to the number of sequences carrying the number of mutations indicated in the periphery of the charts. The total number of independent sequences analysed is indicated in the centre of each chart. **b**, Proportion of Sμ sequences carrying different numbers of mutations

72 h after stimulation with CpG. **c**, Distribution of point mutations. The region sequenced is indicated with the first base corresponding to position 4,600 in the μ region germline sequence (GenBank accession number J00440). H, position of HindIII restriction enzyme sites. Lower-case letters above the line indicate mutations found among the sequenced clones. Each lower-case letter represents an independent mutational event. Hot spots containing mutations are underlined.

adjacent DNA, that the lesions precede CSR, and that they are AID dependent.

It has been proposed that AID initiates somatic hypermutation and CSR by activating an endonuclease that produces lesions in either V genes or switch regions²¹. In this model, CSR breaks would be generated by means of nicking at staggered positions on both strands²¹. We propose that such lesions become associated with Nbs1/ γ -H2AX foci in the G1 phase of the cell cycle and that repair proceeds by NHEJ^{14–16}. By contrast, DNA breaks associated with hypermutation in V genes are found in the S/G2 phase of the cell cycle¹¹ and are repaired by pathways independent of DNA-dependent protein kinase catalytic subunit (DNA-PKcs)²⁷. The lesions in V genes can lead to either gene conversion or somatic hypermutation depending on the mechanism of DNA repair²⁸. In addition to CSR, LPS and IL-4 induce AID-dependent mutations in $\Sigma\mu$. By analogy to V mutation, two alternative pathways might also repair AID-dependent lesions in $\Sigma\mu$. Lesions in $\Sigma\mu$ might be processed by an error-prone polymerase, thereby giving rise to mutation²⁹; alternatively, staggered DSBs could be processed by the NHEJ pathway, leading to CSR^{14–16}. Our observations suggest that AID is either involved directly in producing the switch lesion or activating the switch endonuclease, thereby placing AID upstream of $\Sigma\mu$ mutation, H2AX phosphorylation, Nbs1 recruitment to sites of CSR, and DNA repair. □

Methods

Lymphocyte culture and sorting

B lymphocytes from wild-type (C57BL/6), AID^{-/-} (ref. 1), Ig κ knock-in³⁰ and H2AX^{-/-} mice (Supplementary Information Fig. 1) were isolated from spleen using CD19 microbeads (Miltenyi Biotec). A total of 1×10^6 cells ml⁻¹ was cultured with LPS and IL-4, or 10 nM CpG (MWG biotech, ODN number 1638) for 3 days before isolation. For viable cell DNA staining, 2.5 μ g ml⁻¹ Hoechst 33342 (Molecular Probes) was added to actively growing cells for 2 h, and propidium iodide (0.5 μ g ml⁻¹) was added immediately before laser excitation at 350 nm and 488 nm. Live (propidium-iodide-negative) cells with a DNA content of $2n$ and $>2n$ were electronically sorted using a Vantage SE flow cytometer (Becton Dickinson Biosciences). An aliquot of each of the sorted fractions was fixed with ethanol, stained with propidium iodide (50 μ g ml⁻¹) and re-analysed for DNA content by flow cytometry.

ICC and ICC-FISH analysis

After stimulation, 1×10^6 B cells were spun onto 24-well plates containing 12-mm coverslips (Fisher) coated with 150 μ g ml⁻¹ poly-L-lysine (Sigma). We performed ICC detection essentially as described¹⁰. Briefly, cells were fixed with methanol (-20°C), blocked with 5% goat serum/1% bovine serum albumin (BSA)/1 $\times$ PBS overnight, incubated with either Nbs1 (ref. 10) (1:1,000 dilution), γ -H2AX³ (1:500), Brcal (provided by S. Ganesan; 1:500) or Rad51 (Oncogene; 1:200) polyclonal antibodies, washed and then stained with Alexa-568 conjugated goat anti-rabbit antibody (Molecular Probes). Double staining for Nbs1 with γ -H2AX, Brcal, or Rad51 was performed as described using directly conjugated Nbs1 Alexa-488 antibody¹⁰. For ICC-FISH, antibodies were cross-linked using 50 mM ethylene glycol bis(succinimidyl succinate) for 30 min at 37°C followed by RNase (100 μ g ml⁻¹) treatment for 60 min at 37°C . Chromosomal DNA was denatured in 0.07 M NaOH (pH 13.0) for 2 min followed by immersion in cold PBS. The biotin-labelled DNA probe was hybridized at 37°C overnight, followed by standard FISH washes. DNA probes were detected with avidin-fluorescein isothiocyanate (FITC), and then amplified with biotinylated goat anti-avidin and avidin-FITC (all antibodies diluted 1:200 in 3% BSA/4 $\times$ SSC/0.05% Tween 20; Vector Laboratories).

Mutation analysis

Genomic DNA was amplified by polymerase chain reaction (PCR) from 5,000 cell equivalents in four independent reactions using Expand Long Taq¹⁹ (Roche). This polymerase has a higher error rate than Pfu¹⁹, but the germline $\Sigma\mu$ could not be amplified with Pfu polymerase. We estimate the background error rate of this polymerase to be 0.4×10^{-3} under our amplification conditions based on time 0 values for all sequences analysed (40 mutations in 96,440 bp). For the $\Sigma\mu$, $\text{E}\mu$ and $\text{C}\mu$ regions, amplification conditions were 10 cycles at 94°C (10 s), 60°C (30 s), 68°C (3 min), and 20 cycles at 94°C (10 s), 60°C (30 s), 68°C (3 min and 20 s per cycle). The primers that we used were: μ switch region 5 μ .3 (5'-AATGGATACCTCAGTGGTCTTTAATGGTGGGTGTTA-3') and 3 μ .2 (5'-AGAGGCCTAGATCTGGCTTCTCAAGTAG-3'); μ intronic enhancer 5 μ .E2 (5'-ATTTTAAATGAATTGAGCAATGTTGAGTTGGAGT-3') and 3 μ .EA (5'-GGCAAC TTCAAATTCATTAACACAT-3'); and the Ig μ constant region C μ .1 (5'-AGCCCTC CCCCACCTCCACCTACCTATTAC-3') and C μ .B (5'-ATTCAGGGTTTCATAGGTTGCC AGGTTT-3'). PCR products were cloned using TOPO-TA cloning kit (Invitrogen) and

sequenced using M13 universal primers. Sequence alignment was performed using Sequence manager II software (DNASTAR).

Received 2 October; accepted 2 November 2001.

- Muramatsu, M. *et al.* Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**, 553–563 (2000).
- Carney, J. P. *et al.* The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell* **93**, 477–486 (1998).
- Rogakou, E. P., Boon, C., Redon, C. & Bonner, W. M. Megabase chromatin domains involved in DNA double-strand breaks *in vivo*. *J. Cell. Biol.* **146**, 905–916 (1999).
- Downs, J. A., Lowndes, N. F. & Jackson, S. P. A role for *Saccharomyces cerevisiae* histone H2A in DNA repair. *Nature* **408**, 1001–1004 (2000).
- Revy, P. *et al.* Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* **102**, 565–575 (2000).
- Paull, T. T. *et al.* A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr. Biol.* **10**, 886–895 (2000).
- Li, M. J. *et al.* Rad51 expression and localization in B cells carrying out class switch recombination. *Proc. Natl Acad. Sci. USA* **93**, 10222–10227 (1996).
- Scully, R. *et al.* Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell* **90**, 425–435 (1997).
- Zhu, X. D., Kuster, B., Mann, M., Petrini, J. H. & Lange, T. Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. *Nature Genet.* **25**, 347–352 (2000).
- Chen, H. T. *et al.* Response to RAG-mediated V(D)J cleavage by NBS1 and γ -H2AX. *Science* **290**, 1962–1964 (2000).
- Papavasiliou, E. N. & Schatz, D. G. Cell-cycle-regulated DNA double-stranded breaks in somatic hypermutation of immunoglobulin genes. *Nature* **408**, 216–221 (2000).
- Sale, J. E. & Neuberger, M. S. TdT-accessible breaks are scattered over the immunoglobulin V domain in a constitutively hypermutating B cell line. *Immunity* **9**, 859–869 (1998).
- Bross, L. *et al.* DNA double-strand breaks in immunoglobulin genes undergoing somatic hypermutation. *Immunity* **13**, 589–597 (2000).
- Casellas, R. *et al.* Ku80 is required for immunoglobulin isotype switching. *EMBO J.* **17**, 2404–2411 (1998).
- Manis, J. P. *et al.* Ku70 is required for late B cell development and immunoglobulin heavy chain class switching. *J. Exp. Med.* **187**, 2081–2089 (1998).
- Rolink, A., Melchers, F. & Andersson, J. The SCID but not the RAG-2 gene product is required for $\Sigma\mu$ -Se heavy chain class switching. *Immunity* **5**, 319–330 (1996).
- Stavnezer-Nordgren, J. & Sirlin, S. Specificity of immunoglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching. *EMBO J.* **5**, 95–102 (1986).
- Yancopoulos, G. D. *et al.* Secondary genomic rearrangement events in pre-B cells: VHD/JH replacement by a LINE-1 sequence and directed class switching. *EMBO J.* **5**, 3259–3266 (1986).
- Barnes, W. M. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. *Proc. Natl Acad. Sci. USA* **91**, 2216–2220 (1994).
- Dunnick, W., Hertz, G. Z., Scappino, L. & Gritzmacher, C. DNA sequences at immunoglobulin switch region recombination sites. *Nucleic Acids Res.* **21**, 365–372 (1993).
- Kinoshita, K. & Honjo, T. Linking class-switch recombination with somatic hypermutation. *Nature Rev. Mol. Cell Biol.* **2**, 493–503 (2001).
- Winter, E., Krawinkel, U. & Radbruch, A. Directed Ig class switch recombination in activated murine B cells. *EMBO J.* **6**, 1663–1671 (1987).
- Rogozin, I. B. & Kolchanov, N. A. Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis. *Biochim. Biophys. Acta* **1171**, 11–18 (1992).
- Pasqualucci, L. *et al.* BCL-6 mutations in normal germinal center B cells: evidence of somatic hypermutation acting outside Ig loci. *Proc. Natl Acad. Sci. USA* **95**, 11816–11821 (1998).
- Shen, H. M., Peters, A., Baron, B., Zhu, X. & Storb, U. Mutation of BCL-6 gene in normal B cells by the process of somatic hypermutation of Ig genes. *Science* **280**, 1750–1752 (1998).
- Dunnick, W., Wilson, M. & Stavnezer, J. Mutations, duplication, and deletion of recombined switch regions suggest a role for DNA replication in the immunoglobulin heavy-chain switch. *Mol. Cell. Biol.* **9**, 1850–1856 (1989).
- Bemark, M. *et al.* Somatic hypermutation in the absence of DNA-dependent protein kinase catalytic subunit (DNA-PK(cs)) or recombination-activating gene (RAG)1 activity. *J. Exp. Med.* **192**, 1509–1514 (2000).
- Sale, J. E., Calandrin, D. M., Takata, M., Takeda, S. & Neuberger, M. S. Ablation of XRCC2/3 transforms immunoglobulin V gene conversion into somatic hypermutation. *Nature* **412**, 921–926 (2001).
- Brenner, S. & Milstein, C. Origin of antibody variation. *Nature* **211**, 242–243 (1966).
- Casellas, R. *et al.* Contribution of receptor editing to the antibody repertoire. *Science* **291**, 1541–1544 (2001).

Supplementary Information accompanies the paper on Nature's website (<http://www.nature.com>).

Acknowledgements

We thank F. Rosenthaler for Ig κ probes; S. Sharrow for help with FACS; S. Ganesan for Brcal antibodies; and A. Singer, E. Max, M. Gellert, R. Hodes and E. Besmer for comments on the manuscript and discussions. This work was supported in part by grants from the National Institutes of Health and the Leukemia Society to M.C.N. M.C.N. is a Howard Hughes Medical Institute investigator. The first two authors (S.P. and R.C.) contributed equally to this work.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to A.N. (e-mail: andre_nussenzweig@nih.gov) or M.C.N. (e-mail: nussen@mail.rockefeller.edu).

NEJ1 controls non-homologous end joining in *Saccharomyces cerevisiae*

Maria Valencia*†, Marc Bentele†‡, Moreshwar B. Vaze*, Gernot Herrmann‡§, Eliayhu Kraus*, Sang Eun Lee*§, Primo Schär‡ & James E. Haber*

* Rosenstiel Center and Department of Biology, Brandeis University, Waltham, Massachusetts 02454-9110, USA

‡ Institute of Medical Radiobiology, University of Zürich, CH-8008 Zürich, Switzerland

† These authors contributed equally to this work

Broken DNA ends are rejoined by non-homologous end-joining (NHEJ) pathways requiring the Ku proteins (Ku70, Ku80), DNA ligase IV and its associated protein Lif1/Xrcc4 (ref. 1). In mammalian meiotic cells, Ku protein levels are much lower than in somatic cells, apparently reducing the capacity of meiotic cells to carry out NHEJ and thereby promoting homologous recombination². In *Saccharomyces cerevisiae*, NHEJ is also down-regulated in meiosis-competent *MATa/MATα* diploid cells in comparison with diploids or haploids expressing only *MATa* or *MATα*^{3,4}. Diploids expressing both *MATa* and *MATα* show enhanced mitotic homologous recombination⁴. Here we report that mating-type-dependent regulation of NHEJ in budding yeast is caused in part by transcriptional repression of both *LIF1* and the gene *NEJ1* (YLR265C)—identified from microarray screening of messenger RNAs. Deleting *NEJ1* reduces NHEJ 100-fold in *MATa* or *MATα* haploids. Constitutive expression of *NEJ1*, but not expression of *LIF1*, restores NHEJ in *MATa/MATα* cells. *Nej1* regulates the subcellular distribution of Lif1. A green fluorescent protein (GFP)–Lif1 fusion protein accumulates in the nucleus in cells expressing *NEJ1* but is largely cytoplasmic when *NEJ1* is repressed.

In budding yeast, there are several different NHEJ pathways⁵, all of which require yKu70, yKu80, DNA ligase 4 (Dnl4) and ligase interfacing factor 1 (Lif1) (refs 6–13). When a double-strand break (DSB) is created by transiently expressing the HO endonuclease, the resulting 4-base-pair (bp), 3′-overhanging complementary ends can be efficiently and perfectly rejoined⁴. When HO endonuclease is constitutively expressed, simple re-ligation results in a futile cycle, and survivors appear that have mutated the HO cleavage site by two different pathways⁵—the ends can be misaligned, trimmed and filled in, to produce either 3-bp or 2-bp insertions, or they can be joined at microhomologies to produce a number of different-sized deletions. The perfect ligation pathway and the fill-in pathway both require Mre11, Rad50 and Xrs2 (refs 5, 14). The misalignment pathway seems to be cell-cycle-regulated whereas the deletion pathway does not. All seven NHEJ proteins are involved in the re-ligation of linearized plasmid DNA that is transformed into cells^{6–14}.

The silencing proteins Sir2, Sir3 and Sir4 were also thought to be required for efficient NHEJ¹⁵. Subsequent studies showed, however, that the effect on NHEJ of deleting *SIR* genes was actually an indirect consequence of derepressing the *HMRa* and *HMLα* silent mating-type genes^{3,4}. Thus a Sir[−] haploid cell changes from an *a*- or *α*-mating type expressed at the *MAT* locus to the non-mating phenotype that is also found in *MATa/MATα* diploid cells. Deleting the silent mating-type genes in a Sir[−] haploid cell restored NHEJ to the same level seen in Sir⁺ strains; similarly, creating *MATa/matΔ* or *matΔ/MATα* diploids elevated NHEJ from the low level found in *MATa/MATα* diploids^{3,4}.

Cells expressing both *MATa* and *MATα* produce the *Mata1*–*Matα2* repressor, which turns off transcription of a set of ‘haploid-specific’ genes, including several components of the mating pheromone signalling pathway and the HO endonuclease that regulates *MAT* switching¹⁶. We proposed that the mating-type control of NHEJ should also depend on one or more genes regulated by *Mata1*–*Matα2*.

Although *YKU70*, *YKU80*, *MRE11*, *XRS2* or *DNL4* are not regulated by cell type^{3,17,18} (data not shown), we found that *LIF1* transcription is strongly repressed in diploid *MATa/MATα* strains or haploid *sir2Δ* mutants (Fig. 1a). This is consistent with the presence of a putative binding site for the *Mata1*–*Matα2* repressor, located −175 bp upstream of the *LIF1* open reading frame (ORF). The repression of Lif1 could be sufficient to explain the low level of NHEJ in *MATa/MATα* cells or in *sir* mutants. However, when we overexpressed *LIF1* under the control of a strong inducible promoter (*GAL1*) in a *MATa/MATα* diploid, NHEJ was increased only threefold (Fig. 2), even though this construct will complement *lif1Δ* in a haploid cell. Thus, there must be at least one other as-yet-unknown mating-type-regulated gene needed for NHEJ.

By microarray analysis comparing mRNA isolated from *MATa/MATα*, *MATa/matΔ* and *matΔ/MATα*, we identified a number of new, putative haploid-specific genes (A. P. Gasch, M.B.V., P. O. Brown, D. Botstein and J.E.H., unpublished results). Eight of these candidate genes (*YKL177W*, *YLR040C*, *YLR041W*, *YLR265C*, *YIL117C*, *YGR179C*, *YDR032C*, *TID1* (also known as *RDH54*)) were disrupted and tested for NHEJ. *YLR265C* and *TID1* were also identified in other microarray screens^{17,18}. A galactose-regulated HO endonuclease gene was induced for 1.5 h, after which cells were plated on glucose. In wild-type *MATa HMLα HMRα-B* strains, and in all mutants tested except *YLR265C*, an average of 91.5% of the cells switched from *MATa* to *MATα* or *MATα-B*¹⁹. Because under these conditions greater than 99.9% of *MAT* loci are

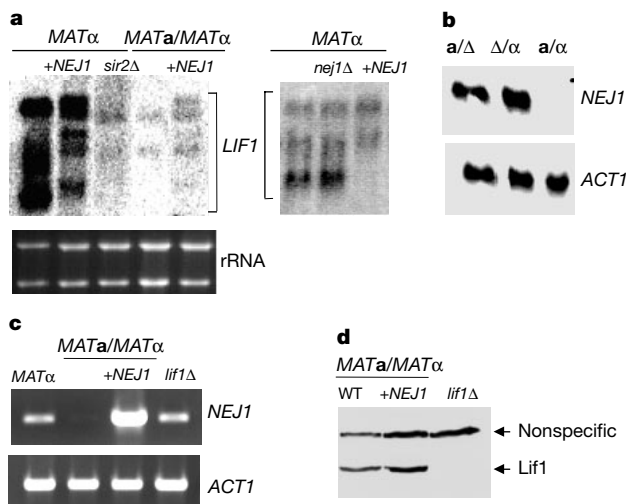


Figure 1 Cell-type regulation of *LIF1* and *NEJ1* mRNA. **a**, Left, comparison of *LIF1* mRNA abundance in haploid *MATα* (FF18734) (with and without a plasmid expressing *NEJ1*) and in a haploid *sir2Δ* derivative, expressing mating-type alleles from *HMLα* and *HMRa*. Also shown is a diploid (*MATa/MATα*) derivative with and without a plasmid conferring constitutive expression of *NEJ1*. Upper panel, autoradiography of *LIF1*-specific mRNA signals; lower panel, ribosomal RNA (rRNA) shows that comparable amounts of RNA were loaded in each lane. Right, *LIF1* mRNA in strain JKM179 and in a *nej1Δ* derivative, plus a strain transformed with an *ADH::NEJ1*-containing plasmid. **b**, *NEJ1* mRNA abundance in haploid *MATa* and diploid *MATa/MATα* cells. Upper panel, autoradiography of *NEJ1*-specific mRNA; lower panel, autoradiography of *ACT1* mRNA. **c**, RT-PCR analysis of *NEJ1* and *ACT1* mRNAs from haploid and diploid JKM179-related cells. **d**, Western blot analysis using a polyclonal antibody against Lif1 that cross-reacts with an unknown protein. WT, wild type.

§ Present address: Department of Dermatology, University of Cologne, D-50931 Cologne, Germany (G.H.); Department of Molecular Medicine/IBT UTHSC-SA, San Antonio, Texas 78245, USA (S.E.L.).

cleaved⁴, the residual 8.5% that had not changed mating type had instead re-ligated the DSB ends to restore the original *MATa* locus. In a *yku70Δ* strain that was defective in NHEJ, less than 0.1% of HO-induced cells retained the *MATa* locus. In *ylr265cΔ* strains none of more than 100 progeny remained *MATa*. This suggested that loss of YLR265C prevented re-ligation of the HO-cut DNA ends by NHEJ.

We then examined the effect of deleting YLR265C on NHEJ in strain JKM179, where both *HML* and *HMR* are deleted, thereby preventing homologous recombination. Continuous expression of HO endonuclease from a galactose-inducible promoter yields about 0.2% survival in wild-type cells and about 100-fold less in strains lacking Ku proteins or Dnl4. Deletion of *YLR265C*, a gene we call here non-homologous end-joining defective (*NEJ1*), impaired NHEJ as severely as *yku70Δ* or *dnl4Δ* (Fig. 3a). The NHEJ defect of *nej1Δ* is also seen in *MATa* haploids assayed by the re-ligation of a linearized, transformed plasmid (Fig. 3b). The effect of *nej1Δ* is as great as that seen for *yku70Δ* and for *sir2Δ* (in this strain *sir2Δ* causes the expression of *Mata1*–*Mata2* from *HMLα* and *HMRα*). There was also a threefold reduction of NHEJ in a *MATa*/*MATα* *nej1Δ*/*nej1Δ* diploid (Fig. 3c), where plasmid end joining is already inefficient because of mating-type regulation. Similarly, homozygous deletion of *DNL4* in a *MATa*/*MATα* diploid caused only a small reduction of NHEJ efficiency (Fig. 2), indicating that mating-type repression of *NEJ1* is nearly complete.

We confirmed by northern blot analysis that, in addition to *LIF1*, *NEJ1* is also strongly mating-type-regulated, being high in *MATa*/*matΔ* or *matΔ*/*MATα* diploids but virtually undetectable in an isogenic *MATa*/*MATα* strain (Fig. 1b). Similar to *LIF1*, *NEJ1* has a consensus-binding site for the *Mata1*–*Mata2* repressor, located –132 bp upstream of the ORF. Expression of *NEJ1* from a constitutive *ADH1* promoter—not regulated by mating type—restored efficient NHEJ to *MATa*/*MATα* diploids in different genetic backgrounds (Figs 2 and 3 and data not shown), and this restoration was fully dependent on functional Dnl4 (Fig. 2).

We next investigated whether Nej1 functions directly at the site of the DSB, as do Dnl4 and Lif1 (ref. 20), or if it is a regulator of other genes or proteins involved in the process. The amino-acid sequence of Nej1 predicts two transmembrane helices in the amino-terminal domain, so that it might be located in a nuclear membrane compartment or in the cytoplasm²¹. However, as expression of Nej1 is sufficient to restore NHEJ ability to diploid cells, it must somehow affect the status of the transcriptionally repressed Lif1. Nej1 might therefore be involved in regulating expression, stability or subcellular localization of Lif1. Over-expressing Nej1 has a qualitative effect on *LIF1* mRNA, causing the accumulation of

species with higher molecular masses (Fig. 1a); however, western blot analysis showed that the level of Lif1 did not change significantly (Fig. 1d). Moreover, when we used microarrays to compare the levels of RNA in *MATa*/*MATα* diploids with and without the plasmid carrying *ADH::NEJ1*, there was no change in the level of *LIF1* mRNA. In fact there was no change in the level of any other *MATa*/*MATα*-regulated gene, nor of any gene involved in NHEJ or homologous recombination (data not shown). These data suggest that Nej1 acts post-transcriptionally to affect Lif1 activity.

We, and others, have found that Nej1 interacts with Lif1 in a two-hybrid screen^{22–25}. Our data further suggest that Nej1 may affect directly the status of Lif1 before its participation with Dnl4 at the site of a DSB²⁰. To test this idea, we cloned full-length *LIF1* into pUG36, fusing the N terminus of the protein to yeast-enhanced GFP (EGFP) under the control of the *MET25* promoter²⁶. When we examined the EGFP–Lif1 fusion protein in haploid cells (Fig. 4a), nearly all the protein was concentrated in the nucleus, as confirmed by 4,6-diamidino-2-phenylindole (DAPI) staining of the nucleus (not shown, but see Fig. 4e). However, in isogenic *MATa*/*MATα* diploids, the preferential nuclear localization was lost, and the GFP signal was spread over the entire cell (Fig. 4d), with a punctuate pattern, which is dependent on the fixation procedure, in the cytoplasm. EGFP–Lif1 is not fully excluded from the nucleus. Western blotting confirmed that the EGFP–Lif1 protein was

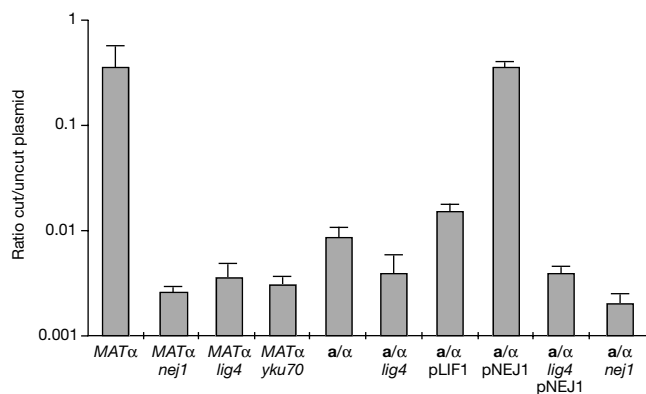


Figure 2 Effect of *LIF1* and *NEJ1* on NHEJ ligation of linearized plasmid DNA transformed into yeast. Results are presented as relative transformation efficiencies (ratios of cut/uncut plasmid) obtained by parallel transformation of *EcoRI*-cleaved and circular plasmid DNA. Error bars represent one standard deviation.

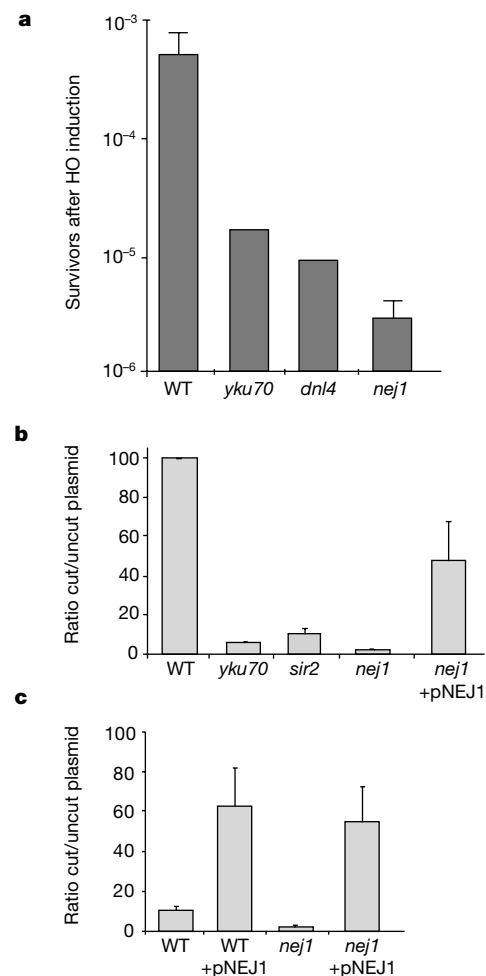


Figure 3 Role of *NEJ1* in chromosomal and plasmid NHEJ. **a**, Effect of *NEJ1* on repair of a chromosomal DSB in strain JKM179 that cannot be repaired by homologous recombination. **b**, Effect of *NEJ1* in haploid cells in the rejoining of plasmid pRS314 cleaved with *Bam*HI. **c**, Effect of *NEJ1* on plasmid end joining in *MATa*/*MATα* diploid cells. WT, wild type. Error bars represent one standard deviation.

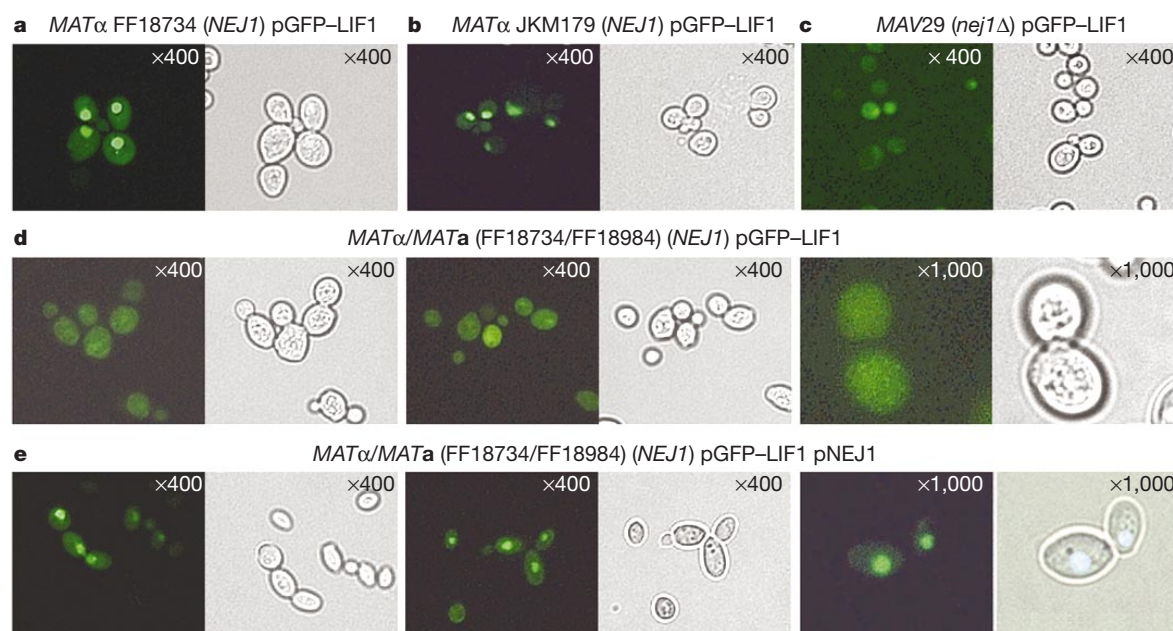


Figure 4 Localization of EGFP-Lif1 in haploid *MATα* and diploid *MATa/MATα* cells. **a**, Localization of EGFP-Lif1 to the nucleus in haploid *MATα* (FF18734) cells. **b, c**, Haploid strain JKM179 (*MATα*) shows nuclear localization of EGFP-Lif1 whereas a *nej1Δ* derivative, MAV29 (**c**), shows loss of preferential localization. **d**, In *MATa/MATα* diploids (FF18734/FF18984), where *NEJ1* is repressed, EGFP-Lif1 is not concentrated in the nucleus but is distributed across the entire cell. **e**, Expression of *NEJ1* from a

galactose-inducible promoter in plasmid pNEJ1 restores nuclear localization to EGFP-Lif1 in most of the cells. In the far-right panel, the differential interference contrast (DIC) image is merged with the DAPI staining of the nucleus (blue), showing co-localization of EGFP-Lif1 and DNA. All cells were grown and analysed under identical conditions. In control cells expressing EGFP only, strong staining of the entire cells is seen (not shown). The level of magnification is indicated.

intact and equally abundant in all cell types analysed. Thus, under conditions where *NEJ1* is repressed, the localization of Lif1 is altered. When these diploid cells were transformed with the plasmid pMV10, expressing *Nej1* from a *GAL1* promoter, EGFP-Lif1 was again preferentially localized to the nucleus in 55% of cells compared with fewer than 4% without expression of *NEJ1* (Fig. 4e).

We confirmed these results by comparing the localization of EGFP-Lif1 in isogenic wild-type and *nej1Δ* cells of a different genetic background (Fig. 4b, c). Here again the localization of EGFP-Lif1 was concentrated in the nucleus in wild-type cells but distributed uniformly in *nej1Δ*. Our results differ from a recent report²³ stating that the presence of *NEJ1* did not affect the localization of Flag-tagged Lif1, which remained predominantly nuclear in *nej1Δ* cells. It is possible that Flag-Lif1 has been altered in such a way that it is found always in the nucleus. In our experiments with two different strains, we see a definitive change in subcellular distribution of EGFP-Lif1 when *Nej1* is expressed. Such redistribution provides strong evidence that *Nej1* does indeed affect the localization of Lif1. This imposes a stringent additional control on NHEJ in which *LIF1* mRNA is repressed but, in addition, the nuclear localization of the residual protein is altered, thus preventing Lif1 from carrying out NHEJ.

We conclude that *Nej1* facilitates the transport of Lif1 into the nucleus, where it can enable Dnl4 to participate at the site of a DSB. Whether *Nej1* can itself be immunoprecipitated with DNA near the site of the DSB, as is the case for Dnl4 and Lif1 (ref. 20), has not been shown; however, *Nej1* could also be important in creating a complex of NHEJ proteins in the nucleus. This might explain why, in the absence of *Nej1*, some EGFP-Lif1 is apparently in the nucleus, although most of the protein is cytoplasmic. The fact that over-expression of *LIF1* in *MATa/MATα* diploids improves NHEJ threefold (Fig. 2) is also consistent with the conclusion that efficient localization of Lif1 requires *Nej1*. We cannot rule out that the localization of other NHEJ proteins is also influenced by *NEJ1*, although *nej1Δ* cells do not have the short-telomere,

ultraviolet-sensitive or methyl-methanesulphonate-sensitive phenotypes associated with the absence of Mre11–Rad50–Xrs2 (data not shown). Using a slot-blot assay to measure the rate of 5' to 3' resection of the end of the HO-induced DSB at *MAT*²⁷, we found that the absence of *NEJ1* does not affect the rate of resection; in our study it behaved as the wild type (data not shown), in contrast to a previous report²³, which found that degradation was slower.

The downregulation of NHEJ in cells expressing *MATa1* and *MATα2* seems to be an understandable reflection of the fact that, in nature, haploid cells are either *MATa* or *MATα* but diploid cells are *MATa/MATα*. Even in the G1 stage of the cell cycle, diploids have a homologous chromosome with which to repair a double-strand break by homologous recombination. Hence, NHEJ is more advantageous in haploid cells where, in G1, repair by homologous recombination is generally not possible. Moreover, in diploids undergoing meiosis, where 50–100 DSBs are induced, it makes sense that NHEJ should be reduced to promote homologous recombination and crossing over. A similar situation seems to occur in mammals, where the abundance of Ku proteins is significantly lower in cells entering meiosis than in somatic cells². Whether the mammalian homologue of Lif1 (Xrcc4) is also regulated has not been determined. □

Methods

Yeast strains and plasmids

Saccharomyces cerevisiae strains used in these experiments included FF18734 (*MATα leu2-3 trp1-289 ura3-52 his7-2 lys1-1*) and FF18984 (*MATa leu2-3 ura3-52 his7-1 lys2-1*)⁶, as well as JKM179 (*MATα hmlΔ::ADE1 hmrΔ::ADE1 ade1 lys5 leu2 ura3-52 trp1Δ::hisG ADE3::GAL::HO*)²⁷. Deletions of *LIF1* (ref. 6), *SIR2* (ref. 4) and *YKU70* (ref. 27) were described previously. The *nej1Δ* deletion was a precise deletion of the ORF, marked by KAN-MX, obtained from Research Genetics. pLIF1 and pNEJ1 are yeast centromeric plasmids YCpIF3 (ref. 6) and pMV01, a derivative of pDB20 (ref. 28) into which the ORF of *LIF1* or *NEJ1* was inserted for expression under the control of the strong *GAL1* and *ADH1* promoters. Details are available on request. EGFP-LIF1 was constructed by fusing the full-length *LIF1* gene to the *MET25* promoter and EGFP sequences in plasmid pUG36 as described previously²⁵.

Growth conditions and NHEJ assays

Cells were grown at 30 °C in YEPD medium or in YEP-lactate to which a final concentration of 2% galactose was added to induce the *GAL::HO* gene²⁷.

Ligation of linearized plasmids with restriction endonuclease was carried out as described previously^{4,11}. NHEJ of an HO-induced DSB on chromosome III was carried out in strain JKM179, as described previously⁴, in which cleavage at the *MAT* locus cannot be repaired by homologous recombination, as *HML* and *HMR* are deleted. Competition between NHEJ and homologous recombination was carried out using derivatives of strain XW652 (*MATa HMLα HMRα-B leu2-3,112 ura3-52 trp1 lys5 ADE3::GALHO*)¹⁹.

RNA and protein analysis

RNA and protein isolation as well as northern and western blots were performed as described previously⁶. Ten micrograms of total RNA were separated on 1% denaturing agarose gels before northern blotting and hybridization with *LIF1*- and *NEJ1*-specific radioactive probes. Polymerase chain reaction with reverse transcription (RT-PCR) of *NEJ1* RNA was carried out with a Superscript II RT-PCR kit, according to the manufacturer's instructions. Primer sequences and details are available on request. Microarray analysis of mRNA in wild-type and *nej1Δ* strains was carried out in the laboratory of P. Brown and D. Botstein²⁹.

Microscopic analysis

GFP staining was analysed with living and formaldehyde-fixed (3.7%, 15 min at room temperature) cells taken from late log phase. Both methods produced essentially the same results. Shown are examples of formaldehyde-fixed cells. We carried out microscopy on a LEICA microscope with 'PL Fluotar' objectives (×40 oil/×100 oil). Images were captured with a digital camera 'LEICADC200' and processed with Adobe Photoshop.

Received 12 October; accepted 8 November 2001.

- Jeggo, P. A. DNA breakage and repair. *Adv. Genet.* **38**, 185–218 (1998).
- Goedecke, W., Eijpe, M., Offenberg, H. H., van Aalderen, M. & Heyting, C. Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis. *Nature Genet.* **23**, 194–198 (1999).
- Åström, S. U., Okamura, S. M. & Rine, J. Yeast cell-type regulation of DNA repair. *Nature* **397**, 310 (1999).
- Lee, S. E., Pâques, F., Sylvan, J. & Haber, J. E. Role of yeast *SIR* genes and mating type in channeling double-strand breaks to homologous and nonhomologous recombination pathways. *Curr. Biol.* **9**, 767–770 (1999).
- Moore, J. K. & Haber, J. E. Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**, 2164–2173 (1996).
- Herrmann, G., Lindahl, T. & Schar, P. *Saccharomyces cerevisiae* LIF1: a function involved in DNA double-strand break repair related to mammalian XRCC4. *EMBO J.* **17**, 4188–4198 (1998).
- Wilson, T. E., Grawunder, U. & Lieber, M. R. Yeast DNA ligase IV mediates non-homologous DNA end joining. *Nature* **388**, 495–498 (1997).
- Tsukamoto, Y., Kato, J. & Ikeda, H. Hdf1, a yeast Ku-protein homologue, is involved in illegitimate recombination, but not in homologous recombination. *Nucleic Acids Res.* **24**, 2067–2072 (1996).
- Milne, G. T., Jin, S., Shannon, K. B. & Weaver, D. T. Mutations in two Ku homologs define a DNA end-joining repair pathway in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**, 4189–4198 (1996).
- Boulton, S. J. & Jackson, S. P. Identification of a *Saccharomyces cerevisiae* Ku80 homologue: roles in DNA double strand break rejoining and in telomeric maintenance. *Nucleic Acids Res.* **24**, 4639–4348 (1996).
- Schär, P., Herrmann, G., Daly, G. & Lindahl, T. A newly identified DNA ligase of *Saccharomyces cerevisiae* involved in *RAD52*-independent repair of DNA double-strand breaks. *Genes Dev.* **11**, 1912–1924 (1997).

- Teo, S. H. & Jackson, S. P. Identification of *Saccharomyces cerevisiae* DNA ligase IV: involvement in DNA double-strand break repair. *EMBO J.* **16**, 4788–4795 (1997).
- Boulton, S. J. & Jackson, S. P. *Saccharomyces cerevisiae* Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways. *EMBO J.* **15**, 5093–5103 (1996).
- Tsukamoto, Y., Kato, J. & Ikeda, H. Effects of mutations of *RAD50*, *RAD51*, *RAD52*, and related genes on illegitimate recombination in *Saccharomyces cerevisiae*. *Genetics* **142**, 383–391 (1996).
- Tsukamoto, Y., Kato, J. & Ikeda, H. Silencing factors participate in DNA repair and recombination in *Saccharomyces cerevisiae*. *Nature* **388**, 900–903 (1997).
- Haber, J. E. Mating-type gene switching in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **32**, 561–599 (1998).
- Galitski, T., Saldanha, A. J., Styles, C. A., Lander, E. S. & Fink, G. R. Ploidy regulation of gene expression. *Science* **285**, 251–254 (1999).
- Wyrick, J. J. *et al.* Chromosomal landscape of nucleosome-dependent gene expression and silencing in yeast. *Nature* **402**, 418–421 (1999).
- Wu, X. & Haber, J. E. A 700 bp *cis*-acting region controls mating-type dependent recombination along the entire left arm of yeast chromosome III. *Cell* **87**, 277–285 (1996).
- Teo, S. H. & Jackson, S. P. Lif1p targets the DNA ligase Lig4p to sites of DNA double-strand breaks. *Curr. Biol.* **10**, 165–168 (2000).
- Nakai, K. & Horton, P. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem. Sci.* **24**, 34–36 (1999).
- Ito, T. *et al.* A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc. Natl Acad. Sci. USA* **98**, 4569–4574 (2001).
- Kegel, A., Sjöstrand, J. O. & Åström, S. U. Nej1p, a cell type-specific regulator of nonhomologous end joining in yeast. *Curr. Biol.* **11**, 1611–1617 (2001).
- Ooi, S. L., Shoemaker, D. D. & Boeke, J. D. A DNA microarray-based genetic screen for nonhomologous end-joining mutants in *Saccharomyces cerevisiae*. *Science*, 8 November 2001 (10.1126/science.1065961).
- Frank-Vaillant, M. & Marcand, S. NHEJ regulation by mating type is exercised through a novel protein, Lif2p, essential to the Ligase IV pathway. *Genes Dev.* **15**, 3005–3012 (2001).
- Cormack, B. P. *et al.* Yeast-enhanced green fluorescent protein (yEGFP) a reporter of gene expression in *Candida albicans*. *Microbiology* **143**, 303–311 (1997).
- Lee, S. E. *et al.* *Saccharomyces* Ku70, Mre11/Rad50 and RPA proteins regulate adaptation to G2/M arrest DNA damage. *Cell* **94**, 399–409 (1998).
- Becker, D. M., Fikes, J. D. & Guarente, L. A cDNA encoding a human CCAAT-binding protein cloned by functional complementation in yeast. *Proc. Natl Acad. Sci. USA* **88**, 1968–1972 (1991).
- Gasch, A. P. *et al.* Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* **11**, 4241–4257 (2000).

Acknowledgements

We are grateful for collaboration with A. Gasch, P. Brown and D. Botstein to analyse mRNA abundance under various conditions, and for their generosity in declining to be authors on this paper. S.E.L. is a Hildegard A. Becher Foundation fellow of The Leukemia and Lymphoma Society. G.H. was supported by the Deutsche Forschungsgesellschaft (DFG). This work was supported by a grant of the Swiss National Science Foundation to M.B. and P.S. and DOE and National Institutes of Health grants to J.E.H.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to J.E.H. (e-mail: haber@brandeis.edu) or P.S. (e-mail: schaer@imr.unizh.ch).

Life and death in the cell

Microscopes to view living cells — and kits to detect dying cells.

ALEXIS **PARP-1 and its Homologs**

The cDNA encoding human poly(ADP-ribose) polymerase (PARP) was cloned in 1987 by several groups independently [1,2]. An additional 'Selected Review Articles' with the discovery of new members (homologs) of the PARP family, PARP-2 is now referred to as PARP-1. The expressed gene encoding human PARP-1 is located on chromosome 14p11-q12 [1,2].

Recent publications have reported the isolation of partial (1A) and full-length (1B) cDNAs for human and mouse homologs of PARP-1. These genes, termed PARP-2, are a 64kDa protein that contains a nuclear localization signal (NLS) and is activated by DNA breaks, although its DNA-binding domain is very different from that of PARP-1.

Table 1: Characteristics of the various mammalian homologs of PARP-1

Species	Gene	Accession No.	Size (kDa)	Isotype
Human	PARP-1	U05401	113.2	1A, 1B
Human	PARP-2	U05402	64.0	1A, 1B
Human	PARP-3	U05403	113.2	1A, 1B
Human	PARP-4	U05404	113.2	1A, 1B
Human	PARP-5	U05405	113.2	1A, 1B
Human	PARP-6	U05406	113.2	1A, 1B
Human	PARP-7	U05407	113.2	1A, 1B
Human	PARP-8	U05408	113.2	1A, 1B
Human	PARP-9	U05409	113.2	1A, 1B
Human	PARP-10	U05410	113.2	1A, 1B
Human	PARP-11	U05411	113.2	1A, 1B
Human	PARP-12	U05412	113.2	1A, 1B
Human	PARP-13	U05413	113.2	1A, 1B
Human	PARP-14	U05414	113.2	1A, 1B
Human	PARP-15	U05415	113.2	1A, 1B
Human	PARP-16	U05416	113.2	1A, 1B
Human	PARP-17	U05417	113.2	1A, 1B
Human	PARP-18	U05418	113.2	1A, 1B
Human	PARP-19	U05419	113.2	1A, 1B
Human	PARP-20	U05420	113.2	1A, 1B
Human	PARP-21	U05421	113.2	1A, 1B
Human	PARP-22	U05422	113.2	1A, 1B
Human	PARP-23	U05423	113.2	1A, 1B
Human	PARP-24	U05424	113.2	1A, 1B
Human	PARP-25	U05425	113.2	1A, 1B
Human	PARP-26	U05426	113.2	1A, 1B
Human	PARP-27	U05427	113.2	1A, 1B
Human	PARP-28	U05428	113.2	1A, 1B
Human	PARP-29	U05429	113.2	1A, 1B
Human	PARP-30	U05430	113.2	1A, 1B
Human	PARP-31	U05431	113.2	1A, 1B
Human	PARP-32	U05432	113.2	1A, 1B
Human	PARP-33	U05433	113.2	1A, 1B
Human	PARP-34	U05434	113.2	1A, 1B
Human	PARP-35	U05435	113.2	1A, 1B
Human	PARP-36	U05436	113.2	1A, 1B
Human	PARP-37	U05437	113.2	1A, 1B
Human	PARP-38	U05438	113.2	1A, 1B
Human	PARP-39	U05439	113.2	1A, 1B
Human	PARP-40	U05440	113.2	1A, 1B
Human	PARP-41	U05441	113.2	1A, 1B
Human	PARP-42	U05442	113.2	1A, 1B
Human	PARP-43	U05443	113.2	1A, 1B
Human	PARP-44	U05444	113.2	1A, 1B
Human	PARP-45	U05445	113.2	1A, 1B
Human	PARP-46	U05446	113.2	1A, 1B
Human	PARP-47	U05447	113.2	1A, 1B
Human	PARP-48	U05448	113.2	1A, 1B
Human	PARP-49	U05449	113.2	1A, 1B
Human	PARP-50	U05450	113.2	1A, 1B
Human	PARP-51	U05451	113.2	1A, 1B
Human	PARP-52	U05452	113.2	1A, 1B
Human	PARP-53	U05453	113.2	1A, 1B
Human	PARP-54	U05454	113.2	1A, 1B
Human	PARP-55	U05455	113.2	1A, 1B
Human	PARP-56	U05456	113.2	1A, 1B
Human	PARP-57	U05457	113.2	1A, 1B
Human	PARP-58	U05458	113.2	1A, 1B
Human	PARP-59	U05459	113.2	1A, 1B
Human	PARP-60	U05460	113.2	1A, 1B
Human	PARP-61	U05461	113.2	1A, 1B
Human	PARP-62	U05462	113.2	1A, 1B
Human	PARP-63	U05463	113.2	1A, 1B
Human	PARP-64	U05464	113.2	1A, 1B
Human	PARP-65	U05465	113.2	1A, 1B
Human	PARP-66	U05466	113.2	1A, 1B
Human	PARP-67	U05467	113.2	1A, 1B
Human	PARP-68	U05468	113.2	1A, 1B
Human	PARP-69	U05469	113.2	1A, 1B
Human	PARP-70	U05470	113.2	1A, 1B
Human	PARP-71	U05471	113.2	1A, 1B
Human	PARP-72	U05472	113.2	1A, 1B
Human	PARP-73	U05473	113.2	1A, 1B
Human	PARP-74	U05474	113.2	1A, 1B
Human	PARP-75	U05475	113.2	1A, 1B
Human	PARP-76	U05476	113.2	1A, 1B
Human	PARP-77	U05477	113.2	1A, 1B
Human	PARP-78	U05478	113.2	1A, 1B
Human	PARP-79	U05479	113.2	1A, 1B
Human	PARP-80	U05480	113.2	1A, 1B
Human	PARP-81	U05481	113.2	1A, 1B
Human	PARP-82	U05482	113.2	1A, 1B
Human	PARP-83	U05483	113.2	1A, 1B
Human	PARP-84	U05484	113.2	1A, 1B
Human	PARP-85	U05485	113.2	1A, 1B
Human	PARP-86	U05486	113.2	1A, 1B
Human	PARP-87	U05487	113.2	1A, 1B
Human	PARP-88	U05488	113.2	1A, 1B
Human	PARP-89	U05489	113.2	1A, 1B
Human	PARP-90	U05490	113.2	1A, 1B
Human	PARP-91	U05491	113.2	1A, 1B
Human	PARP-92	U05492	113.2	1A, 1B
Human	PARP-93	U05493	113.2	1A, 1B
Human	PARP-94	U05494	113.2	1A, 1B
Human	PARP-95	U05495	113.2	1A, 1B
Human	PARP-96	U05496	113.2	1A, 1B
Human	PARP-97	U05497	113.2	1A, 1B
Human	PARP-98	U05498	113.2	1A, 1B
Human	PARP-99	U05499	113.2	1A, 1B
Human	PARP-100	U05500	113.2	1A, 1B

PARP life: the mini-catalogue from Alexis.

PARP Mini-Catalogue

Alexis Biochemicals www.alexis-corp.com

Full marks for apoptosis detection

Poly (ADP-ribose) polymerase (PARP) synthesis is a useful parameter for identifying apoptotic cells. This new mini-catalogue features PARP homologues, antisera and antibodies to the various forms of PARP, and PARP inhibitors. Two technical notes, 'PARP-1 and its homologs' and 'Monoclonal antibody 10H to PAR (A marker for detection of apoptosis and DNA damage)' are included. PARP-2 has recently been found to contain a nuclear localization signal and to be activated by DNA breaks, even though its DNA-binding domain is very different from that of PARP-1.

Mem-PER Protein Extraction Kit

Pierce www.piercenet.com

Rich offerings

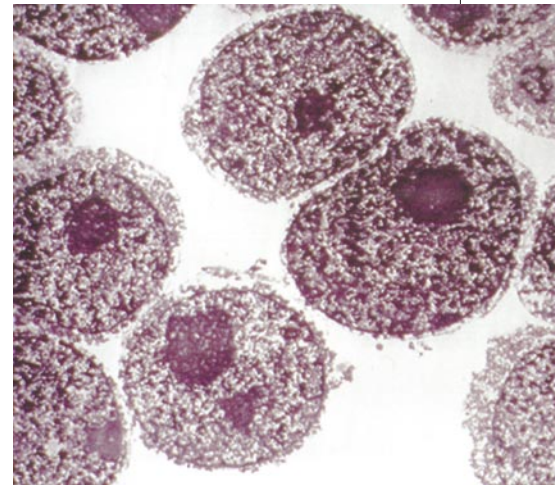
This kit has been designed to produce mammalian cell lysates enriched for membrane proteins in one hour. It consists of three reagents developed for the enrichment of integral and attached membrane proteins obtained from cultured mammalian cells. Cross-contamination of hydrophilic proteins into the hydrophobic (membrane protein) fraction is claimed to be minimal, typically <10%. Applications include initial purification and preparation of proteins for downstream applications, including SDS-PAGE, Western blotting and protein assays.

tion and preparation of proteins for downstream applications, including SDS-PAGE, Western blotting and protein assays.

Nuclei EZ Prep and PURE Prep

Sigma-Aldrich www.sigma-sial.com
Soft and sweet

The Nuclei EZ Prep isolation kit can be used with both suspension and adherent mammalian tissue culture cells. Simultaneous harvest and lysis minimizes artefacts due to sample handling time for adherent mammalian cell lines. For 'difficult' cells, the PURE Prep kit is suitable for preparation of fragile and pure nuclei from mammalian cell lines and solid tissues. Ultracentrifugation through a dense sucrose cushion protects nuclei and strips away cytoplasmic contaminants. The sucrose cushion concentration can be tailored to specific needs. The kits can be used as a source of nuclear components, to produce nuclei for *in vitro* apoptosis assays, to examine transcriptional status of cells, and in proteomic studies.



In suspension: Jurkat cells, by PURE Prep.

Eclipse TE2000

Nikon www.nikonusa.com
Live cell microscopy

Available in three models, Nikon's TE2000 inverted research microscopes all feature a multi-port design and a stratum structure which allows them to be flexibly configured. All models are able to use multiple optical accessories either separately or simultaneously, supporting multiple input illumination sources as well as output detector functions. Multiple epifluorescence techniques may then be used simultaneously or sequentially, allowing other techniques such as high-resolution deconvolution 3D images which may be incorporated together with FRAP or FRET applications. Laser illumination techniques such as FRAP (fluorescence recovery after photobleaching), tweezers or laser microdissection

DermaTACS

Trevigen www.trevigen.com
Superficial knowledge

Trevigen's new *in situ* apoptosis detection kit was developed for use in skin research where distinct identification of cells with fragmented DNA is required. The DermaTACS kit contains the reagents required for detection of apoptosis. TACS-Nuclease is provided with the kit for generating a positive control with the researcher's own samples. EpiDerm control slides, which are sections of synthetic human skin with and without UVB irradiation, are also available.

ADVERTISEMENTS

BACs • YACs • PACs cDNAs

We provide clones and colony membranes for a number of human, mouse and rat libraries as well as a custom screening service for the libraries.

ResGen™
Invitrogen Corporation

U.S. or Canada 800-533-4363
FAX 256-536-9016

Check out our web site at
www.resgen.com

CUSTOMISED
immunoassays for
research and production

BioGenes
Gesellschaft für Biopolymere mbH

Internet www.biogenes.de
Berlin *Germany
Phone +49 (0)30-65 76 23 96

are readily utilized. Nikon's stray light Noise Terminator makes it possible to capture images with maximized signal-to-noise ratios. The TE2000E is a high-precision Z-focus automated model that features five output ports and five-way motorized light path changer. It is intended for advanced research requiring image capture in 3D, including confocal microscopy and deconvolution processing. The TE2000U is a universal model with four output ports as standard, plus an optional fifth user-defined position to give a five-way light path. The U model also features an integrated magnification changer module providing $1\times$ or $1.5\times$ magnification to all ports. The TE2000S is a basic model that can be used for specific tasks and is equipped with two output ports as standard.



Nikon's flexible TE2000.

Axioplan 2 MOT Imaging E microscope

Zeiss

www.zeiss.com

Split-second live imaging

This variation on the Axioplan theme features a high-speed fluorescence shutter, motorized double TV tube, removable field/aperture diaphragm slider and a dual-control knob system with motorized z-axis to facilitate motor control. With an optimized light path, an integrated high-speed shutter allows short exposure times in fluorescence of as little as 20 milliseconds, thereby protecting specimens against bleaching in live cell imaging. A new motor control enables fast, vibration-free positioning of objectives, reflector modules and filter wheels to prevent pixel shift in multi-channel fluorescence. Z-resolution, a major factor in successful 3D imaging techniques such as deconvolution, is said to be improved. The motorized double TV tube allows image recording with two cameras, with manual or software-controlled neutral or dichroic beam splitting. A third camera can be attached via the double video adapter.

Fas ligand vesicles

Upstate Biotechnology

www.upstatebiotechnology.co.uk

Affordable, bioactive Fas ligand (FasL)

Upstate's new preparation of FasL is claimed to exert a much higher cytotoxic and apoptotic activity than that of soluble FasL (sFasL), cell-associated FasL, and anti-Fas mAb (*J. Immunol.*, 164, 5062–5069; 2000). Fas (CD95) ligand (FasL) is a member of the TNF superfamily expressed on various cell types including T cells. When FasL cross-links Fas of Fas-expressing cells, an apoptotic program is activated leading to cell death. Membrane bound FasL (catalogue no. 01-210) has the unique cytotoxic ability of killing targets that are resistant to sFas and anti-Fas mAb and thus represents a new and powerful biological agent in killing Fas-expressing cells. The apoptotic activity of membrane bound FasL is unique in that it can be further

enhanced in response to Polybrene, and poly-L-lysine unlike cell-associated FasL, sFasL or anti-Fas mAb which are not affected.

Luminex Reagents

Biosource International www.biosource.com

Multiplex cytokine antibody kits

Th1/Th2 and pro-inflammatory cytokine multiplex reagents for Luminex LabMap100 systems are now available from Biosource. Antibody-bead kits allow quantitation of mouse or human IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN- γ and TNF- α in one, 50 μ l sample. These are the only kits validated for serum and plasma analysis on the market today and are also suited for TCS samples. Highly sensitive kits detect protein levels below 10 pg per ml, with results in under 4 hours. NIBSC standard calibrated cytokine kits ensure accurate measurement of protein levels. Antibody-bead kits are sold in a single analyte format for flexible parameter choice and are optimized for one species-specific buffer kit to facilitate easy multiplexing.

Human IFN- γ ELISpot

Bender Medsystems

www.bendermedsystems.com

Antigen response detection to a T

This ELISpot is marketed as a highly specific and sensitive method of analysing T-cell responses to individual antigens (Th1 or Th2). It is sensitive to one singular antigen-specific T-cell out of 1,000 irrelevant cells. This means a single cell producing interferon- γ can be detected. The IFN- γ ELISpot kit features high-affinity antibodies and produces clearly differentiated spots. It is suitable for studies of immunological processes associated with viral infections, allergies, autoimmune diseases and neoplastic disorders. Each kit includes sufficient reagents for five 96-well microtitre plates.

These notes are compiled in the Nature office from information provided by the manufacturers.

ADVERTISEMENTS

Proteasome inhibition?

- ~ epoxomicin ~
- ~ PR39 ~ lactacystin ~
- ~ clasto-lactacystin ~ β ~
- ~ lactone ~ P131 ~
- ~ MG-132 ~ MG-262 ~

www.proteasome.com

Affiniti Research Products Ltd.
Mamhead Castle, Exeter, EX6 8HD, U.K.
T.+44(0) 1626 891010 – F+44(0) 1626 891090
– Email info@proteasome.com –

**DIVERSE
SMALL
MOLECULES
READY FOR
SCREENING**



**10K–50K
Compounds
in Plates**

- Next Day Delivery*
- Competitively Priced
- *(Some Restrictions Apply)

Chembridge Corporation • 800.964.6143
858.451.7400 • 858.451.7401 FAX • E-mail: chem@chembridge.com

www.chembridge.com

Silver Stain Kit II for Electrophoresis

**Rapid, sensitive detection
of proteins & nucleic acids**

Wako

Wako BioProducts
Wako Chemicals USA, Inc.
1600 Bellwood Road
Richmond, VA 23237
Telephone: (804) 271-7677
Facsimile: (804) 271-7791
(800) 992-9256
bioproducts@wakousa.com

Contacts

Publisher: Fabien Savenay
Editor: Paul Smaglik
Sales Director: Ben Crowe

European Head Office, London

The Macmillan Building
4 Crinan Street
London N1 9XW, UK
Tel +44 (0) 20 7843 4961
Fax +44 (0) 20 7843 4996
e-mail: naturejobs@nature.com

Group European Manager:

Leonie Welss (4954)

European Manager:

Nevin Bayouni (4978)

UK/ RoW/ Ireland:

Matt Powell (4953), Ben Corp (4974),
Andy Douglas (4975)

Holland/ Italy: Nevin Bayouni (4978)

Scandinavia: Silje Opstrup (4994)

Spain/ Portugal: Leonie Welss
(4954)

Production Manager:

Billie Franklin

To send films and materials use

London address above.

Tel +44 (0) 20 7843 4814

Fax +44 (0) 20 7843 4996

e-mail: naturejobs@nature.com

International

Advertising Coordinator:

Laura Pearson (4977)

Naturejobs web development:

Tom Hancock

Naturejobs online production:

Ben Lund

European Satellite Offices

France/ Belgium:

Christine Niox-Chateau

Tel +33 (0) 1 43 20 16 51

Fax +33 (0) 1 43 20 51 52

e-mail: c.nioxchateau@nature.com

Germany/ Austria/ Switzerland:

Patrick Phelan/ Kate Turner

Tel +49 89 54 90 57 11/-2

Fax +49 89 54 90 57 20

e-mails: p.phelan@nature.com

k.turner@nature.com

US Head Office, New York

345 Park Avenue South,
10th Floor, New York, NY 10010-1707

Tel +1 800 989 7718

Fax +1 800 989 7103

e-mail: naturejobs@natureny.com

US Sales Director:

Ben Crowe

US Sales Manager:

Payton Mason

US Advertising Coordinator:

Corissa Salzman

Japan Head Office, Tokyo

MIG Ichigaya Building (5F),

19-1 Harakata-machi,

Shinjuku-ku,

Tokyo 162-0841

Tel +81 3 3267 8751

Fax +81 3 3267 8746

e-mail: k.cowan@naturejp.com

Japan Manager:

Kate Cowan

naturejobs

Lessons from literature

With the Nobel prizes celebrating their centenary this year, the science awards have received a lot of attention. But on the eve of next week's presentation ceremony, perhaps it is time to look at the literature prize — especially the 1930 award won by Sinclair Lewis in part for his story on immunologist Martin Arrowsmith.

Arrowsmith's career path is neither smooth nor easy — but it offers some useful lessons. At medical school, he apprentices under a professor of bacteriology, who is a stickler for methodology. After a sabbatical, he returns as a disciple to the dean, who is an expert at pacifying people (lesson 1: choose your adviser wisely).

After going into practice, Arrowsmith finds it difficult to balance medicine and research — a public-health position proves to be more politics than science. And a pathology post turns out to be repetitive and dull (lesson 2: if you're committed to research, avoid other options).

His old methodology mentor helps Arrowsmith find some solace at a well-funded private institute (lesson 3: networking pays). But, free of patients and politics, he is instead challenged by economics and ethics when he has to choose whether to freely administer a new vaccine against bubonic plague or to conduct a controlled, blinded trial. When his decision ends in equal parts success and tragedy, Arrowsmith and a friend retreat to a small lab in Vermont, ostensibly for the purity of basic science. But even then, they must establish a business to fund their work (lesson 4: there is no such thing as a free lunch).

Arrowsmith, with all his sector-hopping, seems like a twenty-first-century scientist. His ultimate career lesson — that every position, no matter how promising, comes with its share of frustrations and trade-offs — certainly still resonates.

Paul Smaglik

Naturejobs editor



Contents

SPECIAL REPORT

Assisting astronomy's
rising stars

p4

WWW.NATUREJOBS.COM

Career centre
Information on the scientific
job market

FOCUS

SPOTLIGHT

RECRUITMENT

SCIENTIFIC ANNOUNCEMENTS

SCIENTIFIC EVENTS

As astronomy shifts towards automation and collaboration, the nature of employment in the field is also changing, says Leslie Sage.



There has been no shortage of investment in astronomy since 1989. By 2004, close to US\$2 billion will have been spent worldwide on large ground-based optical observatories. Four space-based telescopes will have been launched for over \$10 billion. And numerous planetary missions will have flown, including the expensive Galileo mission to Europa and Cassini's trip to Saturn.

A generation from now, such investments could well be viewed as a 'golden age' of astronomy. Although this might be true for hardware and missions, for most of that time it hasn't extended to employment. The early 1980s through to 1999 are likely to be remembered as a bleak time for PhD astronomers looking for tenure-track academic jobs.

But is the investment in technology turning the employment tide? New missions and observatories have already created new jobs at the postdoc level, and these may eventually provide long-term jobs — although the resulting positions might be different from the academic positions young astronomers have historically pursued.

For example, astronomers used to visit a telescope for a few days or weeks, collect their data and then go home. Now, more and more get their data remotely, relying on technicians at the facilities. The new large telescopes coming online will mostly require remote observing, or even 'service observing', in which a local employee collects the data and passes them on to the astronomer. Space-based observatories always do this, and some level of processing is often done before the astronomer even sees the data. More data will also be archived for public access, which will lead to research projects that involve no new observations.

In addition, an increasing number of astronomers are involved in large collaborations. "For years, our model of success has been the brilliant scientist who toiled on independent research to the exclusion of all else, aggressively defended their results, and had a PhD adviser marketing them for a faculty position," says Andrea Schweitzer, chair of the American Astronomical Society (AAS) committee on employment. "Now the astronomical community needs to reassess what talents and personalities will produce the science in the twenty-first century. How shall we modify our filters so that intelligent scientists who have broad knowledge, good communication

skills and a spirit of teamwork stand out above the noise?"

This is true across the physical and biological sciences — if anything, astronomy is behind the curve. Yet there are still enough small groups for there to have been little evolution in the way that young researchers are assessed for advancement. This may be one of the greatest challenges to be faced over the next 10–15 years, as the criterion of primary publications becomes less relevant for hiring decisions, and the need for team players who can work within the constraints of large groups becomes more important (see 'Surviving collaborations', opposite).

MIXED SIGNALS

There are some indications that an investment in people commensurate with the investment in facilities is beginning, says Steve Beckwith, director of the Space Telescope Science Institute in Baltimore, Maryland. The November 2001 AAS job register lists 44 faculty jobs, another 10 for long-term support positions, and many more postdoc openings.

But graduate students and postdocs wonder whether this is a temporary situation. Today's astronomy graduate students are more pessimistic about their employment prospects than those who entered grad school in the early to mid-1980s. But many grad students still hope for a job either as a faculty member or as a researcher in one of the many institutes that have sprung up recently. Will there be growth in the number of faculty jobs? The signals right now are mixed.

On the bright side, Bruce Margon, associate director for science at the Space Telescope Science Institute, says that the end of mandatory retirement in the United States over the past decade has not changed retirement patterns dramatically. Moreover, there is increasing interest among the two-year and four-year colleges in hiring astronomers.

Technology may be a boon to these jobs, Margon points out. For example, the National Virtual Observatory (NVO) continues the democratization of astronomy that began with the establishment of the national observatories about 40 years ago. Someone with a high-end Unix workstation at a small college will have the same access to NVO data as senior members of large departments at bigger universities. What will be needed is talent in sophisticated

Bruce Margon sees opportunities for talented programmers to deal with the rising amounts of data being collected.





Surviving collaborations

programming to extract the data from the collection.

On the other hand, Peter Strittmatter, director of the Steward Observatory at the University of Arizona in Tucson, sees little potential for new growth in faculty jobs.

But although there may not be growth, there is almost always demand, says Virginia Trimble of the University of California, Irvine. "There have always been more people than jobs, but more jobs than outstanding people, and, for whatever reason, no department ever seems to place an ad that says: 'The University of Realistic Expectations expects to appoint an average astronomer, who will get an occasional grant and not actually be stoned by irate students.'"

So what are realistic expectations for young astronomers, and how should they maximize their chances for getting stable, long-term employment?

SERVICE CHARGE

One solution may be to redefine what stable, long-term employment means. The traditional concept — tenure-track faculty position — may not be the most viable for many young astronomers. As the field moves towards bigger telescopes and larger collaborations, service positions — essential for collecting the data at big telescopes, and for doing the processing needed for collaborations — are becoming more important. These jobs require a lot of experience and judgement, and will generally provide stable employment for PhD astronomers, although probably with little or no time formally allocated for the employee's own research. The trade-off of greater stability early in a career — and of a job more likely to be 9 to 5 — has to be judged against the risk of disappointment later in life, as fellow students rise through the faculty ranks.

A path not considered by many students, but which has historically been one to success, is to specialize in instrumentation. Numerous panels from, and studies by, the AAS show that there is a constant demand for people working in instrumentation. Although Strittmatter and Beckwith agree that there is a traditional bias against hiring such people for faculty jobs, there are many other career opportunities open to them, particularly at major facilities.

Code development for the numerical simulations that are increasingly part of front-line research may be the 'new instrumentation'. But Adam Burrows, a

Large collaborations are creating jobs — but young astronomers need to ensure that they don't get lost in the shuffle.

This is especially true for postdocs, who must show leadership if they are to use their jobs as a way to land a more permanent position. They must make part of a large project identifiably their own, or expand it in a new direction that is uniquely their own, says Steve Beckwith, director of the Space Telescope Science Institute in Baltimore.

Postdocs must assert themselves if they want a traditional faculty job because the people who will interview them will consider that to be a

good characteristic.

But Charles Alcock, an astronomer at the University of Pennsylvania in Philadelphia, notes that distinguishing oneself in a large collaboration can be hard — especially if you're junior. The field is very conservative and tends to look for publications on which a job applicant is first author. But once the collaboration exceeds a certain size, there may simply not be enough good results to go around and give everyone a first-author paper. And important contributions may be made in technical, rather than scientific areas, so someone may not get the recognition they deserve. **L.S.**

Steve Beckwith (right) encourages young astronomers to make their mark.



Lost in the crowd: Steve Beckwith notes that life in a large group can prove difficult (left).

Peter Strittmatter (below) feels there is little chance that faculty will expand.

theoretical astrophysicist at the University of Arizona, cautions that faculty jobs generally are open only to those who think deeply about the physics, and who push the research in new directions. There probably will not be many programming jobs associated with the NVO, though, because the consortium has decided that programming should come from highly developed existing sources, as well as from the information technology and mathematics communities.

Leslie Sage is *Nature's* astronomy editor.

American Astronomical Society ♦ www.aas.org

National Virtual Observatory ♦ www.srl.caltech.edu/nvo

